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(71)(72) Applicants and Inventors: EGLI, Margaret, A. [US/US]; 705 Heinel Circle, Roseville, MN 55113 (US). GROH, Susanne [US/US]; 2384 Larpenter Avenue, #7, Falcon Heights, MN 55113 (US). KIANIAN, Shahryar, F. [US/US]; Apartment 230, 3201 20th Street South, Fargo, ND 58104 (US). PHILLIPS, Ronald, L. [US/US]; 819 Tanglewood Drive, Shoreview, MN 55126 (US). RINES, Howard, W. [US/US]; 4292 Nancy Place, Shoreview, MN 55126 (US). SOMERS, David, A. [US/US]; 924 West County Road B2, Roseville, MN 55113 (US).

(54) Title: DNA ENCODING OAT ACETYL CoA CARBOXYLASE

(57) Abstract

The present invention provides the complete cDNA sequence of maize acetyl CoA carboxylase and methods for conferring herbicide tolerance and/or altering the oil content of plants by introducing and expressing a plant acetyl CoA carboxylase gene in plant cells. The method of imparting herbicide tolerance to a plant includes the steps of introducing an expression cassette encoding a plant acetyl CoA carboxylase or an antisense DNA sequence complementary to the sequence for a plant acetyl CoA carboxylase gene operably linked to a promoter functional in plant cells, into the cells of a plant tissue and expressing the plant acetyl CoA carboxylase gene in an amount effective to render the acetyl CoA carboxylase and/or plant cell tolerant to the herbicides. The method of altering the oil content in a plant includes the steps of introducing an expression cassette into plant cells and expressing the acetyl CoA carboxylase gene in an amount effective to alter the oil content of the cells. The expression cassette can also be introduced into other host cells to increase yield of a plant acetyl CoA carboxylase so that crystallized enzyme can be used to screen and identify other herbicides that bind to and inhibit the enzyme. Also provided is a partial DNA sequence encoding oat acetyl CoA carboxylase.

FATTY ACID BIOSYNTHESIS

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DNA ENCODING OAT ACETYL CoA CARBOXYLASE

Background of the Invention

Acetyl CoA carboxylase (ACCase) is an enzyme involved in
many important metabolic pathways in plant, animal and bacterial cells. The
enzyme is especially important in fatty acid synthesis in plants and is sensitive to

5 inhibition by some types of herbicides. Structurally, ACCases are biotinylated
and are quite large enzymes consisting of one or more subunits. For example,
most ACCases of animals, higher plants, and yeast are dimers of 420 to 700 kD
native MW and contain subunits of 200 to 280 kD. Diatom and algal ACCases
are 700 to 740 kD tetramers of 160 to 180 kD subunits. Bacterial ACCase

10 consists of three dissociable proteins; biotin carboxylase (51 kD), biotin carboxyl
carrier protein (22.5 kD), and biotin transcarboxylase (130 kD).

Acetyl CoA Carboxylase (ACCase) catalyzes the ATP-dependent
formation of malonyl-CoA from acetyl-CoA and bicarbonate in animal, plant,
and bacterial cells. Malonyl-CoA is an essential substrate for (i) de novo fatty

15 acid (FA) synthesis, (ii) fatty acid elongation, (iii) synthesis of secondary
metabolites such as flavonoids and anthocyanins, and (iv) malonylation of some
amino acids and secondary metabolites. Synthesis of malonyl-CoA is the first
committed step of flavonoid and fatty acid synthesis and current evidence
suggests that ACCase catalyzes the rate-limiting step of fatty acid synthesis.

20 Formation of malonyl-CoA by ACCase occurs via two partial reactions and
requires a biotin prosthetic group:

(i)E-biotin + ATP + HCO_3 --> E-biotin-CO_2 + ADP + Pi

(ii)E-biotin-CO_2 + Acetyl-CoA --> E-biotin + malonyl-CoA

(NET)Acetyl-CoA + ATP + HCO_3 --> malonyl-CoA + ADP + Pi
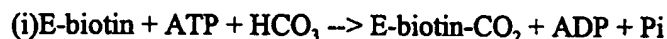
25 In E. coli, these reactions are catalyzed by three distinct components; biotin
carboxylase, biotin transcarboxylase, and biotin carboxyl carrier protein, which
can be separated and yet retain partial activity. Plant and animal ACCases
contain all three activities on a single polypeptide.

In plants, most ACCase activity is located in plastids of green and

30 non-green plant tissues including leaves and oil seeds. Leaf ACCase activity is
primarily located in mesophyll cells, but lesser amounts have been found in C-4

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bundle sheath cells and in epidermal cells. The subcellular location of ACCase

activity in epidermal cells is　unknown, but since synthesis of very long-chain

fatty acids (VLCFA) for formation of waxes, cutin, and suberin occurs on the

endoplasmic reticulum (ER), malonyl-CoA might also be derived from a

5　　cytosolic ACCase. In contrast, rat ACCase is primarily cytosolic or associated

with the outer mitochondrial membrane.

De novo fatty acid synthesis in chloroplasts involves successive

2-carbon additions to acetate, using malonate as the 2-C donor. All

intermediates are attached to acyl carrier protein (ACP). Synthesis in plastids

10　　resembles that in E. coli in that the fatty acid synthesis complex can be

dissociated into separate enzymes: β-ketoacyl-ACP synthase (KAS), β-ketoacyl-

ACP reductase, β-hydroxyl-ACP dehydratase, and enoyl-ACP reductase,

acetyl-CoA:ACP transacylase, and malonyl-CoA:ACP transacylase. A highly

active KASIII isozyme catalyzes the condensation of acetyl-CoA and

15　　malonyl-ACP. Successive additions of malonyl-CoA to acyl-ACPs catalyzed by

KAS I form C16 acyl-ACP, some of which is converted to C18 acyl-ACP by

KAS II and then to C18:1-ACP. Fatty acid metabolism then diverges; de-

esterification allows movement to the cytoplasm (eukaryotic path) where fatty

acids may be further unsaturated and/or elongated by additions of malonyl-CoA

20　　in the ER. Alternatively, fatty acids are linked to glycerol-3-phosphate

(prokaryotic path), further unsaturated, and used for synthesis of chloroplast

lipids. A portion of cytoplasmic lipids returns to the chloroplast. The relative

contributions of these two paths are species-specific but appear to be relatively

flexible in mutants blocked in either path. In oil-storing organs such as

25　　cotyledons and monocot embryos, the triacylglycerides are stored in cytoplasmic

oil bodies surrounded by a single unit membrane.

Condensation of malonyl-CoA with phenylpropionyl-CoAs or

acetyl-CoA leads to synthesis of flavonoids, anthocyanins, or to polyacetates.

Condensation is increased by light, elicitors, or pathogens and may be the rate-

30　　limiting step in synthesis of some phytoalexins. In addition to the secondary

metabolites derived by de novo synthesis, malonyl conjugates of flavonoid

glycosides, formed by malonyl-CoA:flavonoid glycoside malonyltransferase,

D-amino acids and 1-amino-carboxyl-cyclopropane (ethylene precursor) are

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found in plants. Malonylated compounds accumulate in vacuoles, probably after synthesis in the cytoplasm.

An important property of ACCase is the central role it plays in fatty acid synthesis and accumulation in plants and seeds. Available evidence

5   supports the idea that ACCase activity is the rate-limiting step for de novo fatty acid synthesis in plants. High rates of ACCase activity in vitro parallel or slightly precede high rates of lipid deposition or [^{14}C]acetate incorporation into lipids in developing leaves and oil seeds. Significant changes in plant ACCase activity occur during chloroplast development and increase in ACCase activity

10   correlates with lipid deposition in developing oil seeds. Turnham et al., Biochem. J., 212:223 (1883); and Beittenmiller et al., Plant Physiol., 100:923 (1992).

Among other properties, ACCase in most monocots is also inhibited by several herbicides. [^{14}C]acetate incorporation into maize lipids is

15   strongly inhibited by fluazifop and sethoxydim due to inhibition of plastid ACCase. In barley, however, fluazifop had little effect on [^{14}C]acetate incorporation into very long-chain fatty acids. Since synthesis of very long-chain fatty acids occurs in the cytosol on the ER, and de novo fatty acid synthesis occurs in the plastids, cytosolic malonyl-CoA might be supplied by a

20   herbicide insensitive ACCase isozyme.

There are three general mechanisms by which plants may be resistant to, or tolerant of, herbicides. These mechanisms include insensitivity at the site of action of the herbicide (usually an enzyme), rapid metabolism (conjugation or degradation) of the herbicide, or poor uptake and translocation of

25   the herbicide. Altering the herbicide site of action from a sensitive to an insensitive form is the preferred method of conferring tolerance on a sensitive plant species. This is because tolerance of this nature is likely to be a dominant trait encoded by a single gene, and is likely to encompass whole families of compounds that share a single site of action, not just individual chemicals.

30   Therefore, detailed information concerning the biochemical site and mechanism of herbicide action is of great importance and can be applied in two ways. First, the information can be used to develop cell selection strategies for the efficient identification and isolation of appropriate herbicide-tolerant variants. Second, it

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can be used to characterize the variant cell lines and regenerated plants that result from the selections.

Tissue culture methods have been used to select for resistance (or tolerance) using a variety of herbicides and plant species (see review by

5   Meredith and Carlson, 1982, in Herbicide Resistance in Plants, eds. Lebaron and Gressel, pp. 275-291, John Wiley and Sons, NY). For example, P. C. Anderson et al., in U.S. Patent No. 4,761,373, disclose the use of tissue culture methods to produce maize plants resistant to herbicidal imidazolidones and sulfonamides. The resistance is due to the presence of altered acetohydroxy acid synthase

10   which is resistant to deactivation by these herbicides.

Certain 1,3-cyclohexanediones exhibit general and selective herbicidal activity against plants. One such cyclohexanedione is sethoxydim {2-[1-(ethoxyimino)-butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one}. Sethoxydim is commercially available from BASF (Parsippany, New

15   Jersey) under the designation POAST™.

Other herbicidal cyclohexanediones include clethodim, (E,E)-(±)-2-[1-[[(3-chloro-2-propenyl)oxy] imino]propyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one; available as SELECT™ from Chevron Chemical (Valent) (Fresno, California); cloproxydim, (E,E)-2-[1-[[(3-chloro-2-

20   propenyl)oxy]imino] butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one; available as SELECTONE™ from Chevron Chemical (Valent) (Fresno, California); and tralkoxydim, 2-[1-(ethoxyimino)propyl]-3-hydroxy-5-mesitylcyclohex-2-enone, available as GRASP™ from Dow Chemical USA (Midland, Michigan).

25   For purposes of reference in the present specification, the herbicides described in the two preceding paragraphs and other structurally related herbicidal compounds, are collectively referred to as the cyclohexanedione family of herbicides.

Certain aryloxyphenoxypropanoic acids exhibit general and

30   selective herbicidal activity against plants. In these compounds, the aryloxy group may be phenoxy, pyridinyloxy or quinoxalinyl. One such herbicidal aryloxyphenoxypropanoic acid is haloxyfop, {2-[4-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]- propanoic acid}, which is available

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as VERDICT™ from Dow Chemical USA (Midland, Michigan). Another is
diclofop, {(±)-2-[4-(2,4-dichlorophenoxy)-phenoxy]propanoic acid}, available as
HOELON™ from Hoechst-Roussel Agri-Vet Company (Somerville, New
Jersey).

5   Other members of this family of herbicides include fenoxyaprop,
(±)-2-[4-[(6-chloro-2-benzoxazolyl)oxy] phenoxy]propanoic acid; available as
WHIP™ from Hoechst-Roussel Agri-Vet Company (Somerville, New Jersey);
fluazifop, (±)-2-[4-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic
acid; available as FUSILADE™ from ICI Americas (Wilmington, Delaware);

10   fluazifop-P, (R)-2-[4-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic
acid; available as FUSILADE 2000™ from ICI Americas (Wilmington,
Delaware); and quizalofop, (±)-2-[4[(6-chloro-2-quinoxalinyl)-
oxy]phenoxy]propanoic acid; available as ASSURE™ from E. I. DuPont de
Nemours (Wilmington, Delaware).

15   For purposes of reference in the present specification, the
herbicides referred to in the two preceding paragraphs and other structurally
related herbicidal compounds, are collectively referred to as herbicidal
aryloxyphenoxypropanoic acids.

          Thus, there is a need for methods to develop plants that are

20   resistant or tolerant to herbicides. There is also a need to increase the oil and/or
fatty acid content of the plants and seeds, as well as for methods to increase their
resistance to herbicides. Moreover, there is a need to identify and clone genes
important in conferring herbicide tolerance and in increasing the oil content of
plants.

25

Summary of the Invention

          The present invention provides an isolated and purified DNA
molecule comprising a DNA segment encoding a plant acetyl CoA carboxylase
such as a monocot acetyl CoA carboxylase, e.g., a maize or oat acetyl CoA

30   carboxylase, and methods for conferring herbicide tolerance and/or altering the
oil content of plants by introducing and expressing a plant acetyl CoA
carboxylase gene in the plant cells. The DNA molecule encoding a plant acetyl
CoA carboxylase can encode an unaltered plant acetyl CoA carboxylase or an

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altered plant acetyl CoA carboxylase substantially tolerant to inhibition by cyclohexanedione or aryloxyphenoxypropanoic acid herbicides as well as encoding an antisense DNA sequence that is substantially complementary to a plant acetyl CoA carboxylase gene or to a portion thereof. A DNA molecule of

5 the invention can also further comprise an amino terminal plant chloroplast transit peptide sequence operably linked to the plant acetyl CoA carboxylase gene. The DNA molecule of the invention preferably encodes the plastidic form of plant acetyl CoA carboxylase. The invention also provides for probes, e.g., a probe which is detectably labeled or which binds to a detectable label, or

10 primers, e.g., a single-strand DNA molecule having at least seven nucleotide bases, that hybridize to a DNA encoding a plant acetyl CoA carboxylase, e.g., a DNA molecule comprising SEQ ID NO:23 or its complement. For example, the present invention provides an isolated and purified DNA molecule of at least seven nucleotide bases which hybridizes under high stringency conditions to a

15 DNA molecule comprising a DNA segment encoding a plant acetyl CoA carboxylase and provides a hybridization probe comprising an isolated and purified DNA molecule of at least seven nucleotide bases, which is detectably labeled or which binds to a detectable label, which DNA molecule hybridizes under high stringency conditions to the non-coding strand of a DNA molecule

20 comprising a DNA segment encoding a plant acetyl CoA carboxylase. High stringency conditions are defined as: hybridization at 65°C for at least 16 hours in 5xSSC, 1x Denhardt's solution, 50 mM Tris-HCl, pH 8, 0.2% SDS, 10 mM EDTA, 0.1 mg/ml salmon sperm DNA, followed by washing twice for 5 minutes in 2xSSC, 0.5% SDS at 25°C, once for 10 minutes in 0.2xSSC, 0.1% SDS at

25 25°C and twice for 30 minutes in 0.2xSSC, 0.1% SDS at 65°C.

The method of imparting cyclohexanedione or aryloxyphenoxypropanoic acid herbicide tolerance to a plant includes the steps of introducing a chimeric or recombinant DNA molecule comprising a gene coding for a plant acetyl CoA carboxylase or an altered or a functional mutant

30 thereof operably linked to a promoter functional in a plant cell into cells of a susceptible plant, and regenerating the transformed plant cells to provide a differentiated plant. The promoter can be an inducible or tissue specific promoter or provide for overexpression of at least about a 2-fold amount of a

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native plant acetyl CoA carboxylase. The functional linkage of a promoter to the
chimeric DNA molecule results in an expression cassette. Expression of the
chimeric DNA molecule is in an amount effective to render the acetyl CoA
carboxylase and/or the plant tissue substantially tolerant to the herbicides relative

5   to the native acetyl CoA carboxylase present in said plant. Herbicide tolerance
can be achieved in the plants by at least two methods, including increasing the
level of gene expression of a native or unaltered acetyl CoA carboxylase, or by
introducing an altered gene coding for an acetyl CoA carboxylase that is less
sensitive to herbicide inhibition. The level of gene expression can be increased

10   by either combining a plant acetyl CoA carboxylase gene with a promoter that
provides for a high level of gene expression, such as a 35S cauliflower mosaic
virus promoter (CaMV), or by introducing multiple copies of the gene into the
cell so that the multiple copies of the gene are integrated into the genome of
transformed plant cells. The preferred plant cells into which to introduce the

15   expression cassette of the invention, to achieve herbicide tolerance, are monocot
plant cells. Preferably, the cells are not oat cells. Once transformed cells
exhibiting herbicide tolerance are obtained, transgenic plants and seeds can then
be regenerated therefrom, and evaluated for stability of the inheritance of the
herbicide tolerance trait.

20   The invention also provides a method for altering, e.g., lowering
or raising, the oil content in a plant. As described hereinbelow, a locus having a
major effect on oat (*Avena sativa* L.) groat oil content was located on linkage
group 11 by quantitative trait locus (QTL) dissection methods. A partial oat
acetyl-CoA carboxylase (ACCase) cDNA with >84% identity to plastidic

25   ACCase sequences from wheat or maize was linked to this locus. Similar QTL
and ACCase placements were obtained with two recombinant inbred populations
(n ≥ 137) with one common parent. The locus on linkage group 11 linked to the
ACCase locus accounted for up to 48% of the phenotypic variance for groat oil
content. Other loci were identified which accounted for an additional 14% of the

30   phenotypic variance. The major locus plus additional QTLs represent three
potentially homoeologous genomic segments in oat that can be followed by as
few as three RFLP markers to produce a large shift in groat oil content. Oat
acetyl CoA carboxylase DNA is also useful in molecular marker-assisted

selection to facilitate breeding high- or low-oil oat, genetic dissection of the
groat oil trait, and to identify specific genes with large effects whose activity
may be manipulated via genetic transformation.

Thus, the invention provides a method which includes the steps of
5   introducing a chimeric or recombinant DNA molecule comprising a gene coding
for a plant, e.g., oat, acetyl CoA carboxylase or an altered or a functional mutant
thereof operably linked to a promoter functional in a plant cell into the cells of
plant tissue and expressing the gene in an amount effective to alter the oil
content of the plant cell. An alteration in oil content can include a change in
10   total oil content over that normally present in that type of plant cell, a change in
the location of the oil in the plant, plant tissue, seed or plant part, and/or a
change in the type of oil present in the cell. For example, low-oil oat includes,
but is not limited to, an oat plant, plant part, plant tissue or groat having about 8
to about 40, preferably about 8 to about 30, and more preferably about 20 to
15   about 30, percent dry weight of oil relative to the corresponding parental, e.g.,
nontransformed or inbred, oat plant, plant part, plant tissue or groat. High-oil oat
includes, but is not limited to, an oat plant, plant part, plant tissue or groat having
about 2 to about 8, preferably 1 to about 4, and more preferably about 2 to about
4, percent dry weight of oil relative to the corresponding parental oat plant, plant
20   part, plant tissue or groat.

An alteration in oil content in the plant cell can be achieved by at
least two methods including:

(1)   an increase or decrease in expression of an unaltered or
altered plant acetyl CoA carboxylase gene; or

25   (2)   by introducing an unaltered, altered or functional mutant
plant acetyl CoA carboxylase gene.

The level of gene expression of an unaltered plant acetyl CoA carboxylase gene
can be increased by either combining an unaltered plant acetyl CoA carboxylase
with a promoter that provides for a high level of gene expression, or by
30   introducing multiple copies of an expression cassette into cells so that multiple
copies of the gene are integrated into the genome. When an altered or a
functional mutant plant acetyl CoA carboxylase gene codes for an enzyme that
exhibits an increase in specific activity, it can lead to an increase in total oil

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content of the plant cell. When an altered or a functional mutant acetyl CoA
carboxylase gene codes for an enzyme having a decrease in specific activity, it
may lead to a decrease in the total oil content of the plant cell. Preferably, the
expression cassette is introduced into dicot plants such as soybeans, canola, and

5   sunflower. In an especially preferred version, transformed cells exhibiting about
a 1.2- to 5-fold increase in total oil content and/or expression or specific activity
of acetyl CoA carboxylase are selected for and used to generate transgenic plants
and seeds exhibiting a substantial increase in oil content. A substantial increase
in oil content depends on the oil content normally present in the plant or seed

10   and can range from about a 1.2 to a 20-fold increase.

        The invention also provides for a method of producing plant
acetyl CoA carboxylase in a host cell. The method includes the steps of
introducing an expression cassette comprising a chimeric gene encoding a plant
acetyl CoA carboxylase or an altered or a functional plant acetyl CoA

15   carboxylase operably linked to a promoter into a host cell and expressing the
gene in an amount sufficient to permit crystallization of the plant acetyl CoA
carboxylase. An expression cassette can include a promoter that is functional in
either a eukaryotic or a prokaryotic cell. Preferably, the expression cassette is
introduced into a prokaryotic cell, such as E. coli, that is routinely used for

20   production of recombinantly produced proteins. Recombinantly produced and
crystallized plant acetyl CoA carboxylase can then be used to identify other
herbicides and that bind to and inhibit acetyl CoA carboxylase in plants. In
addition, the availability of large amounts of purified enzyme can permit the
screening of the efficacy of such herbicides in terms of their ability to bind to, or

25   otherwise inhibit, the activity of the enzyme.

        The present invention also provides a method of introducing an
exogenous plant acetyl CoA carboxylase gene into a host cell comprising
transforming host cells *in vitro* with an expression cassette comprising a
chimeric or recombinant DNA molecule encoding a plant acetyl CoA

30   carboxylase gene operably linked to a promoter functional in the host cell,
expanding the transformed host cells *in vitro*, and identifying a transformed host
cell which expresses the chimeric DNA molecule.

10

Further provided is a method to identify a plant, e.g., an oat plant, having altered characteristics, e.g., altered groat oil content. The method comprises contacting a probe comprising at least a portion of a nucleic acid sequence encoding oat acetyl CoA carboxylase with a sample from an oat plant

5   in an amount sufficient to form complexes, wherein the sample comprises plant genomic nucleic acid. The amount of complex formation is then detected or determined. Preferably, the plant cells in the sample are monocot plant cells, e.g., wheat, rice, maize, barley, millet (e.g., foxtail or finger), sorghum, sugarcane, rye, oat or timothy cells. Also preferably, the DNA molecule encodes

10   a polypeptide comprising SEQ ID NO:24, e.g., a DNA molecule comprising SEQ ID NO:23. Thus, the invention provides a nucleic acid fragment useful in marker-assisted breeding programs such as those which identify plants having altered groat oil content or composition.

Also provided is an expression cassette comprising an

15   endosperm-specific transcriptional control region, e.g., the Dy10 or zein transcriptional control region, operably linked to a DNA encoding a plant acetyl CoA carboxylase, or subunit thereof. Other endosperm-specific promoters are also useful in the compositions and methods of the invention. These promoters include, but are not limited to, ltr1 promoter, a synthetic promoter with

20   endosperm box of HOD (Vincentecarbajosa et al., Plant J., 13, 629 (1998)); rice glutelin gene promoter (Sindhu et al., Plant Sci., 130, 189 (1997); Yoshihara et al., Plant Sci., 121, 63 (1996)); barley alpha-amylase inhibitor subunit promoter (Gross et al., Plant Mol. Biol., 34, 331 (1997)); gamma zein promoter (Towent et al., Plant Mol. Biol., 34, 139 (1997)); maize opaque-2 locus promoter (Rossi et

25   al., J. Biol. Chem., 272, 13758 (1997); Gallusci et al., Mol. & Gen. Genet., 244, 391 (1994)); the zmGBS, zmZ27, osAGP or osGT1 promoter (Russell et al., Transgenic Res., 6, 157 (1997)); alpha globulin promoter (Nakase et al., Plant Mol. Biol., 33, 513 (1997)); hordein promoter (Sorenson et al., Mol. & Gen. Genet., 250, 750 (1996)); Viciafaba legumin and vicilin storage protein gene

30   promoter (Wobus et al., J. Plant Physiol., 145, 592 (1995)); rice waxy gene promoter (Hirano et al., Plant & Cell Physiol., 36, 37 (1995)); oat globulin gene osglo5 promoter (Schubert et al., Plant Mol. Biol., 26, 203 (1994)); napA

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promoter (Stalberg et al., Plant Mol. Biol., 23, 671 (1993)); and rice prolamin

gene promtoer (Zhou et al., Science in China, 36, 1307 (1993)).

The term "consists essentially of" as used with respect to the

present DNA molecules is defined to mean that a major portion of the nucleotide

5   sequence encodes an ACCase, and that nucleotide sequences are not present

which encode proteins other than ACCase or functional equivalents thereof.

Brief Description of the Figures

Figure 1: A schematic depiction of the fatty acid biosynthesis

10   pathway in plants.

Figure 2: A graph depicting the effect of sethoxydim on the

growth of mutant maize callus.

Figure 3: A graph depicting the shoot length growth of maize

seedlings seven days after treatment with sethoxydim.

15   Figure 4: A graph depicting the shoot length growth of maize

seedlings fourteen days after treatment with sethoxydim.

Figure 5: Total soluble and biotinylated polypeptides in ACCase

purification fractions from seedling leaves of maize inbred A619. Proteins were

separated by SDS-PAGE in 7.5% gels and then silver-stained (Panel A). An

20   identical gel was Western-blotted and a longitudinal section of each lane was

probed with avidin (Panel B). Lanes were 1: crude (10 μg); 2: $(NH_4)_2SO_4$

(10 μg); 3: S-300 (5 μg); 4: Blue Sepharose (2 μg); 5: Mono-Q ACCase II

(5 μg); and 6: Mono-Q ACCase I (5 μg). Diagonal lines between lanes indicate

position of molecular weight markers shown on the left.

25   Figure 6: Immunoprecipitation of ACCase activity from B73

leaf, embryo, endosperm, and BMS suspension cultured cells. Equal activities

(0.58 nmol min^{-1}) were incubated with 16 μL serum (immune plus preimmune),

immune complexes were precipitated with Protein A-agarose, and ACCase

activity remaining in the resulting supernatant fraction was measured relative to

30   the preimmune serum control.

Figure 7: Herbicide inhibition of acetyl-(AcCoA) or

propionyl-CoA (Prop-CoA)-dependent $H^{14}CO_3$-incorporation into acid-stable

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product by ACCase I and II Mono-Q fractions. Activities in the presence of haloxyfop (1 μM) are expressed relative to the minus herbicide control.

Figure 8: Comparison of the peptide sequence of maize cDNA clones #15-14 and #18-5 with chicken ACCase. The approximate locations of
5   the biotin carboxylase, biotin binding site, and biotin transcarboxylase functional domains are indicated for the chicken sequence. The percentages of amino acid identity are indicated by cross-hatched boxes for the maize coding sequence. Regions of genomic DNA Type I and Type II clone sequences that align with cDNA #18-5 are indicated by solid heavy lines. The approximate locations of
10 subclone #28 and #16 from genomic Type I and subclone #34 from genomic Type II clones are indicated.

Figure 9: Northern blot of total RNA from leaf, immature embryo and endosperm tissue (16 days after pollination), and Black Mexican Sweet corn (BMS) cells. Lanes contain 10 μg total RNA and were probed with
15 the 2 kb EcoRI fragment of lambda clone #15-14.

Figure 10: DNA sequence (SEQ ID NO. 1) of a 2 kb EcoRI fragment of lambda clone #15-14 including a portion of a maize ACCase gene located at bases 2883 to 83 from the 3' stop codon.

Figure 11: Graph of ACCase activity during seed development in
20 two high oil soybean cell lines and one low oil soybean cell line.

Figure 12: Cloning strategy to obtain the complete coding sequence of the maize ACCase gene.

Figure 13: DNA sequence (SEQ ID NO:5) of a 7470 base pair cDNA of a maize ACCase gene. (Genbank Accession No. U19183).
25   Figure 14: Predicted amino acid sequence of the complete ACCase gene of maize (SEQ ID NO:6).

Figure 15: Restriction map of ACCase genomic clones.

Figure 16: Partial nucleotide sequence of a Type A_1 ACCase genomic clone (SEQ ID NO:12).
30   Figure 17: Partial nucleotide sequence of clone 5A, a Type A ACCase genomic clone (SEQ ID NO:13).

Figure 18: Partial nucleotide sequence of six Type A_2 ACCase genomic clones (A-F) (SEQ ID NOs 14, 15, 16, 17, 18, and 19).

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Figure 19: Partial nucleotide sequence of three Type B ACCase clones (SEQ ID NOs 20, 21 and 22).

Figure 20: SDS/PAGE analysis of chloroplast importation of ^{35}S-labeled ACCase polypeptides. (A) Pea and maize chloroplasts incubated with ^{35}S-labeled ACCase polypeptides for 30 minutes. (B) A time course analysis of the importation of ^{35}S-labeled ACCase polypeptides into maize chloroplasts.

Figure 21: Partial DNA sequence encoding oat ACCase (SEQ ID NO:23, Genbank Accession No. AF072737).

Figure 22: Partial amino acid sequence of an oat ACCase (SEQ ID NO:24).

Figure 23: Scan of test statistics generated by MQTL for the two recombinant inbred populations. A and B are the main effect and QTL × E interaction graphs for the Kanota X Ogle (KO) population, respectively. C and D are the main effect and QTL × E interaction graphs for the Kanota X Marion (KM) population, respectively. Thresholds for type I error rate of 5% after 1000 replications are reported above the graphs.

Figure 24: Histogram of % groat oil concentration for the mean of all environments relative to the number of recombinant inbred lines (RILs) with each parental genotype for the XaccaseA locus. A) The distribution for the Kanota X Ogle population, and B) distribution for the Kanota X Marion population.

Figure 25: Phylogram of ACCases.

Detailed Description of the Invention

The present invention provides a DNA molecule encoding a plant acetyl CoA carboxylase gene and methods for conferring herbicide tolerance and/or altering the oil content of plants by introducing and expressing a plant acetyl CoA carboxylase gene in the plant cells. In plants, acetyl CoA carboxylase plays a central role in regulating fatty acid synthesis and in the sensitivity of monocots to cyclohexanedione or aryloxyphenoxypropanoic acid herbicides.

In accord with the present invention, a plant acetyl CoA carboxylase gene is identified, isolated and combined with a promoter functional

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in a plant cell to provide a recombinant expression cassette. A plant acetyl CoA carboxylase gene can be introduced and expressed in a plant cell. Depending on the type of plant cell, the level of gene expression, and the activity of the enzyme encoded by the gene, introduction of a plant acetyl CoA carboxylase gene into

5   the plant cell can confer herbicide tolerance and/or alteration of the oil of the plant cell.

In monocots, an exogenously introduced plant acetyl CoA carboxylase gene can be expressed at a level effective to render the cells of the plant tissue substantially tolerant to cyclohexanedione or

10   aryloxyphenoxypropanoic acid herbicide levels which normally inhibit a native or endogenous acetyl CoA carboxylase. A native acetyl CoA carboxylase is an enzyme that is normally encoded and expressed in the plant cell prior to transformation. An exogenously introduced plant acetyl CoA carboxylase gene is a gene which has been isolated and amplified from either the same or different

15   type of cell. Exogenous introduction and expression of a plant acetyl CoA carboxylase gene in both monocots and dicots can result in alteration of the oil content and quality of plant tissue and seeds. Exogenous introduction and expression in a host cell, such as a bacteria, can provide for sufficient amounts of plant acetyl CoA carboxylase to allow for crystallization and isolation of the

20   enzyme. Crystallized plant acetyl CoA carboxylase is useful to identify other herbicides that bind to and can inhibit plant acetyl CoA carboxylases. The enzyme could also be used to screen potential herbicidal compounds for efficacy.

A.   **Formation of an Expression Cassette**

An expression cassette of the invention can comprise a chimeric

25   DNA molecule encoding a plant acetyl CoA carboxylase gene or an altered or functional mutant thereof operably linked to a promoter functional in a plant cell. The gene can code for a plant acetyl CoA carboxylase that is substantially tolerant to herbicides, preferably cyclohexanedione and/or aryloxyphenoxypropanoic acid herbicides. An expression cassette of the

30   invention can also include an antisense DNA sequence that is substantially complementary to an acetyl CoA carboxylase gene or a portion thereof operably linked to a promoter functional in a plant cell.

1.   **Isolation and Identification of a Gene Coding for a Plant
      Acetyl CoA Carboxylase**

A gene encoding a plant acetyl CoA carboxylase can be identified
and isolated by standard methods, as described by Sambrook et al., Guide to
Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY (1989). The
gene can be obtained either from monocot or dicot plant cells. When the gene
encoding a plant acetyl CoA carboxylase is obtained from a dicot plant, the
enzyme encoded by the gene exhibits tolerance to cyclohexanedione or
aryloxyphenoxypropanoic acid herbicides. The gene can also be obtained from
herbicide-tolerant maize cell lines, prepared as described in U.S. Patent
No. 5,162,602.

A gene encoding a plant acetyl CoA carboxylase can be identified
by screening of a DNA or cDNA library generated from plant cells. Screening
for DNA fragments that encode all or a portion of the gene encoding a plant
acetyl CoA carboxylase can be accomplished by complementation of an
auxotrophic mutant of acetyl CoA carboxylase in E. coli (fabE) (Bachman,
Microbiological Reviews, 47:180 (1983)) or yeast (accl) (Michionada, Eur. J.
Biochem., 111:79 (1980)) or by screening of plaques for binding to antibodies
that specifically recognize a plant acetyl CoA carboxylase. DNA fragments that
can restore ACCase activity in E. coli or yeast and/or plaques carrying DNA
fragments that are immunoreactive with antibodies to a plant ACCase can be
subcloned into a vector and sequenced and/or used as probes to identify other
cDNA or genomic sequences encoding all or a portion of a plant acetyl CoA
carboxylase gene.

Specific examples of cDNA sequences encoding a portion of a
plant acetyl CoA carboxylase gene include DNA fragments that include a DNA
sequence that substantially corresponds to the coding sequence for the
transcarboxylase active site of a plant acetyl CoA carboxylase, DNA fragments
that include a DNA sequence that substantially corresponds to a coding sequence
for the biotin binding site of a plant acetyl CoA carboxylase, a DNA fragment
encoding the 5' transcriptional start sequence of a plant acetyl CoA carboxylase
gene, and a DNA fragment encoding the 3' transcriptional stop sequence for the
acetyl CoA carboxylase gene. Substantially corresponding DNA sequences

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share about 90% to about 100% DNA sequence homology. Especially preferred
cDNA probes can be obtained from lambda clone #18-5 which include DNA
sequences corresponding to the transcarboxylase active site domain and the
biotin binding site domain. Lambda clone #18-5 includes EcoRI subclones of

5   3.9 kb, 1.2 kb, or 0.23 kb. Lambda subclone #18-5I is an 3.9 kb EcoRI subclone.
The lambda subclone #18-5I has been deposited with the American Type Culture
Collection, Rockville, MD, and given Accession No. 69236.

In a preferred version, a plant acetyl CoA carboxylase gene is
identified and isolated from an herbicide tolerant maize cell line prepared as

10   described in Example II. A cDNA library can be prepared by oligo dT priming.
Plaques containing DNA fragments can be screened with antibodies specific for
maize acetyl CoA carboxylase. DNA fragments encoding a portion of an acetyl
CoA carboxylase gene can be subcloned and sequenced and used as probes to
identify a genomic acetyl CoA carboxylase gene. DNA fragments encoding a

15   portion of a maize acetyl CoA carboxylase can be verified by determining
sequence homology with other known acetyl CoA carboxylases, such as chicken
or yeast acetyl CoA carboxylase, or by hybridization to acetyl CoA carboxylase
specific messenger RNA. Once DNA fragments encoding portions of the 5',
middle and 3' ends as well as the transcarboxylase active site or biotin binding

20   site of a plant acetyl CoA carboxylase are obtained, they can be used to identify
and clone a complete genomic copy of a maize acetyl CoA carboxylase gene.

To isolate a complete copy of a maize acetyl CoA carboxylase
gene, a maize genomic library can then be probed with cDNA probes prepared as
described above. Portions of the genomic copy or copies of a plant acetyl CoA

25   carboxylase gene can be sequenced and the 5' end of the gene are identified by
standard methods including either DNA sequence homology to other acetyl CoA
carboxylase genes or by RNAase protection analysis, as described by Sambrook
et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold
Spring Harbor, New York (1989). Once portions of the 5' end of the gene are

30   identified, complete copies of a plant acetyl CoA carboxylase gene can be
obtained by standard methods, including by cloning or by polymerase chain
reaction (PCR) synthesis using oligonucleotide primers complementary to the
DNA sequence at the 5' end of the gene. The presence of an isolated full-length

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copy of a plant acetyl CoA carboxylase gene can be verified by hybridization, partial sequence analysis, or by expression of a plant acetyl CoA carboxylase enzyme. The maize acetyl CoA carboxylase gene cloned and expressed from a maize herbicide tolerant cell line can be assessed for tolerance to

5   cyclohexanedione or aryloxyphenoxypropanoic acid herbicides by standard methods, as described in Example I.

An expression cassette of the invention can also contain an antisense DNA sequence. A antisense DNA sequence is a sequence that is substantially complementary to all or a portion of a coding sequence of a plant

10   acetyl CoA carboxylase gene. A substantially complementary sequence has about 90% to about 100% DNA sequence homology with that of the coding sequence of all or a portion of a plant acetyl CoA carboxylase. The antisense DNA sequence when expressed can act to inhibit the synthesis and expression of a native plant acetyl CoA carboxylase. Antisense sequences are preferably about

15   200 to 1000 nucleotides long in order to provide sufficient inhibition of synthesis and/or expression of a native acetyl CoA carboxylase. The inhibition of acetyl CoA carboxylase synthesis and gene expression by antisense DNA sequences can be confirmed in a transformed plant cell by standard methods for measuring the presence and/or activity of the enzyme such as described in Examples I and

20   V.

An expression cassette of the invention can also include a functional mutant of a plant acetyl CoA carboxylase gene. Mutants of a plant acetyl CoA carboxylase gene are substantially homologous to a plant acetyl CoA carboxylase gene and are functional if the acetyl CoA carboxylase expressed

25   retains significant enzyme activity. A mutant substantially homologous to a plant acetyl CoA carboxylase can share about 90% to 99.99% DNA sequence with that gene. For example, a mutant acetyl CoA carboxylase gene can code for a herbicide tolerant acetyl CoA carboxylase, or for an acetyl CoA carboxylase with altered substrate specificity so that the total amount of oil content in the

30   plants or seeds is increased, or for an enzyme with an altered substrate specificity so that synthesis of secondary metabolites such as flavonoids or anthocyanins is decreased. A preferred mutant is a gene coding for an acetyl CoA carboxylase

that is substantially tolerant to cyclohexanedione or aryloxyphenoxypropanoic acid herbicide

Altered or functional mutants of a gene coding for a plant acetyl CoA carboxylase can be obtained by several methods. The alteration or

5   mutation of the ACCase gene can be accomplished by a variety of means including, but not limited to, the following methods.

1.   spontaneous variation and direct mutant selection in cultures;

2.   direct or indirect mutagenesis procedures on tissue culture of all cell types, seeds or plants; and

10   3.   mutation of the cloned acetyl CoA carboxylase gene by methods such as site specific mutagenesis (Sambrook et al. cited supra), transposon mediated mutagenesis (Berg et al., Biotechnology, 1:417 (1983)) and deletion mutagenesis (Mitra et al., Molec. Gen. Genetic., 215:294 (1989)).

15   Mutants can be identified by a change in a functional activity of the enzyme encoded by the gene or by detecting a change in the DNA sequence using restriction enzyme mapping or partial sequence analysis.

In a preferred version, a functional mutant gene encoding for a plant acetyl CoA carboxylase tolerant to cyclohexanedione and/or

20   aryloxyphenoxypropanoic acid herbicides is isolated from a maize herbicide tolerant cell line. The maize herbicide tolerant cell line was obtained as described in U.S. Patent No. 5,162,602, issued November 10, 1992, the disclosure of which is incorporated in Examples I-III. Briefly, partially differentiated cell cultures are grown and subcultured with continuous exposures

25   to low herbicide levels. Herbicide concentrations are then gradually increased over several subculture intervals. Maize cells or tissues growing in the presence of normally toxic herbicide levels are repeatedly subcultured in the presence of the herbicide and characterized. Stability of the herbicide tolerance trait of the cultured cells may be evaluated by growing the selected cell lines in the absence

30   of herbicides for various periods of time and then analyzing growth after exposing the tissue to herbicide.

Maize cell lines which are tolerant by virtue of having an altered acetyl CoA carboxylase enzyme can be selected by identifying cell lines having

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enzyme activity in the presence of normally toxic levels of sethoxydim or haloxyfop. The tolerant maize cells can be further evaluated for whether acetyl CoA carboxylase is altered to a less sensitive form or increased in its level of expression.

5   Maize cell lines with a acetyl CoA carboxylase less sensitive to herbicide inhibition can be used to isolate a functional mutant gene of a plant acetyl CoA carboxylase. A DNA library from a maize cell line tolerant to herbicides can be generated and DNA fragments encoding all or a portion of an acetyl CoA carboxylase gene can be identified by hybridization to a cDNA probe

10   encoding a portion of the maize ACCase gene. A complete copy of the altered gene can be obtained either by cloning and ligation or by PCR synthesis using appropriate primers. The isolation of the altered gene coding for acetyl CoA carboxylase can be confirmed in transformed plant cells by determining whether the acetyl CoA carboxylase being expressed retains enzyme activity when

15   exposed to normally toxic levels of herbicides.

        2.   **Promoters**

        Once a plant acetyl CoA carboxylase gene or functional mutant thereof or an antisense DNA sequence is obtained and amplified, it is combined with a promoter functional in a plant cell to form an expression cassette.

20   Most genes have regions of DNA sequence that are known as promoters and which regulate gene expression. Promoter regions are typically found in the flanking DNA sequence upstream from the coding sequence in both procaryotic and eukaryotic cells. A promoter sequence provides for regulation of transcription of the downstream gene sequence and typically includes from

25   about 50 to about 2,000 nucleotide base pairs. Promoter sequences also contain regulatory sequences such as enhancer sequences that can influence the level of gene expression. Some isolated promoter sequences can provide for gene expression of heterologous genes, that is a gene different from the native or homologous gene. Promoter sequences are also known to be strong or weak or

30   inducible. A strong promoter provides for a high level of gene expression, whereas a weak promoter provides for a very low level of gene expression. An inducible promoter is a promoter that provides for turning on and off of gene expression in response to an exogenously added agent or to an environmental or

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developmental stimulus. Promoters can also provide for tissue specific or developmental regulation. An isolated promoter sequence that is a strong promoter for heterologous genes is advantageous because it provides for a sufficient level of gene expression to allow for easy detection and selection of

5   transformed cells and provides for a high level of gene expression when desired.

The promoter in an expression cassette of the invention can provide for overexpression of acetyl CoA of a plant acetyl CoA carboxylase gene or functional mutant thereof. Overexpression of the gene is that amount of gene expression that results in an increase in tolerance of the plant cells to an

10   herbicide or that results in an increase in the total oil content of the cells. Overexpression of an acetyl CoA carboxylase gene is preferably about a 2- to 20-fold increase in expression of an acetyl CoA carboxylase over the expression level of the native acetyl CoA carboxylase. The promoter can also be inducible so that gene expression can be turned on or off by an exogenously added agent.

15   For example, a bacterial promoter such as the P_{tac} promoter can be induced to varying levels of gene expression depending on the level of isothiopropylgalactoside added to the transformed bacterial cells. It may also be preferable to combine the gene with a promoter that provides tissue specific expression or developmentally regulated gene expression in plants.

20   Specific promoters functional in plant cells include the 35S cauliflower mosaic virus promoter, nopaline synthase (NOS) promoter and the like. Currently, a preferred promoter for expression in monocots is the 35S cauliflower mosaic virus promoter.

An acetyl CoA carboxylase gene can be combined with the

25   promoter by standard methods as described in Sambrook cited supra. Briefly, a plasmid containing a promoter such as the 35S cauliflower mosaic virus promoter can be constructed as described in Jefferson, Plant Molecular Biology Reporter, 5,387 (1987) or obtained from Clontech Lab in Palo Alto, CA (e.g. pBI121 or pBI221). Typically these plasmids are constructed to provide for

30   multiple cloning sites having specificity for different restriction enzymes downstream from the promoter. A gene for plant acetyl CoA carboxylase can be subcloned downstream from the promoter using restriction enzymes to ensure that the gene is inserted in proper orientation with respect to the promoter so that

the gene can be expressed. In a preferred version, a maize acetyl CoA
carboxylase is operably linked to a 35 S Came promoter in a plasmid such as
pBI121 or pBI221. Once a plant acetyl CoA carboxylase gene is operably linked
to a promoter and the plasmid, the expression cassette so formed can be

5   subcloned into other plasmids or vectors.

3.   Optional Sequences in the Expression Cassette

The expression cassette can also optionally contain other DNA
sequences. The expression cassette can further be comprised of a chloroplast
transit peptide sequence operably linked between a promoter and a plant acetyl

10   CoA carboxylase gene. If the expression cassette is to be introduced into a plant
cell, the expression cassette can also contain plant transcriptional termination
and polyadenylation signals and translational signals linked to the 3′ terminus of
a plant acetyl CoA carboxylase gene. The expression cassette can also
optionally be further comprised of a plasmid.

15   Because one site of action for biosynthetic pathways involving
plant acetyl CoA carboxylase is the chloroplast, an expression cassette of the
invention can be combined with an exogenous DNA sequence coding for a
chloroplast transit peptide, if necessary. An exogenous chloroplast transit
peptide is one which is not encoded within the plant acetyl CoA carboxylase

20   gene. A chloroplast transit peptide is typically 40 to 70 amino acids in length
and functions post-translationally to direct the protein to the chloroplast. The
transit peptide is cleaved either during or just after import into the chloroplast to
yield the mature protein. The complete copy of a gene encoding a plant acetyl
CoA carboxylase may contain a chloroplast transit peptide sequence. In that

25   case, it may not be necessary to combine an exogenously obtained chloroplast
transit peptide sequence into the expression cassette.

Exogenous chloroplast transit peptide encoding sequences can be
obtained from a variety of plant nuclear genes, so long as the products of the
genes are expressed as preproteins comprising an amino terminal transit peptide

30   and transported into chloroplast. Examples of plant gene products known to
include such transit peptide sequences are the small subunit of ribulose
biphosphate carboxylase, ferredoxin, chlorophyll a/b binding protein, chloroplast
ribosomal proteins encoded by nuclear genes, certain heatshock proteins, amino

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acid biosynthetic enzymes such as acetohydroxy acid synthase, 3-enolpyruvylphosphoshikimate synthase, dihydrodipicolinate synthase, and the like. Alternatively, the DNA fragment coding for the transit peptide may be chemically synthesized either wholly or in part from the known sequences of

5   transit peptides such as those listed above.

Regardless of the source of the DNA fragment coding for the transit peptide, it should include a translation initiation codon and an amino acid sequence that is recognized by and will function properly in chloroplasts of the host plant. Attention should also be given to the amino acid sequence at the

10   junction between the transit peptide and the plant acetyl CoA carboxylase enzyme where it is cleaved to yield the mature enzyme. Certain conserved amino acid sequences have been identified and may serve as a guideline. Precise fusion of the transit peptide coding sequence with the acetyl CoA carboxylase coding sequence may require manipulation of one or both DNA sequences to

15   introduce, for example, a convenient restriction site. This may be accomplished by methods including site directed mutagenesis, insertion of chemically synthesized oligonucleotide linkers and the like.

Once obtained, the chloroplast transit peptide sequence can be appropriately linked to the promoter and a plant acetyl CoA carboxylase gene in

20   an expression cassette using standard methods. Briefly, a plasmid containing a promoter functional in plant cells and having multiple cloning sites downstream can be constructed as described in Jefferson cited supra. The chloroplast transit peptide sequence can be inserted downstream from the promoter using restriction enzymes. A plant acetyl CoA carboxylase gene can then be inserted

25   immediately downstream from and in frame with the 3' terminus of the chloroplast transit peptide sequence so that the chloroplast transit peptide is linked to the amino terminus of the plant acetyl CoA carboxylase. Once formed, the expression cassette can be subcloned into other plasmids or vectors.

When the expression cassette is to be introduced into a plant cell,

30   the expression cassette can also optionally include 3' nontranslated plant regulatory DNA sequences. The 3' nontranslated regulatory DNA sequence preferably includes from about 300 to 1,000 nucleotide base pairs and contains plant transcriptional and translational termination sequence. Specific examples

of 3' nontranslated regulatory DNA sequences functional in plant cells include about 500 base pairs of the 3' flanking DNA sequence of the pea ribulose biphosphate carboxylase small subunit E9 gene, the 3' flanking DNA sequence of the octopine synthase gene, and the 3' flanking DNA sequence of the nopaline

5 synthase gene. These 3' nontranslated regulatory sequences can be obtained as described in An, Methods in Enzymology, 153:292 (1987) or are already present in plasmids available from commercial sources such as Clontech, Palo Alto, CA. The 3' nontranslated regulatory sequences can be operably linked to the 3' terminus of a plant acetyl CoA carboxylase gene by standard methods.

10 An expression cassette of the invention can also be further comprised of a plasmid. Plasmid vectors included additional DNA sequences that provide for easy selection, amplification and transformation of the expression cassette in procaryotic and eukaryotic cells. The additional DNA sequences include origins of replication to provide for autonomous replication of

15 the vector, selectable marker genes, preferably encoding antibiotic resistance, unique multiple cloning sites providing for multiple sites to insert DNA sequences or genes encoded in the expression cassette, and sequences that enhance transformation of prokaryotic and eukaryotic cells. The preferred vectors of the invention are plasmid vectors. The especially preferred vector is

20 the pBI121 or pBI221 vector formed as described by Jefferson cited supra.

Another vector that is useful for expression in both plant and procaryotic cells is the binary Ti vector PGA582. This binary Ti vector has been previously characterized by An, cited supra., and is available from Dr. An. This binary Ti vector can be replicated in procaryotic bacteria such as E. coli and

25 Agrobacterium. The Agrobacterium plasmid vectors can be used to transfer the expression cassette to plant cells. The binary Ti vectors preferably include the nopaline T DNA right and left borders to provide for efficient plant cell transformation, a selectable marker gene, unique multiple cloning sites in the T border regions, the colE1 replication of origin and a wide host range replicon.

30 The binary Ti vectors carrying an expression cassette of the invention can be used to transform both prokaryotic and eukaryotic cells, but is preferably used to transform plant cells.

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B.   Method for Screening for Expression and/or Overexpression of a Plant Acetyl CoA Carboxylase Gene

A method for screening for expression or overexpression of a

5   plant acetyl CoA carboxylase gene is also provided by the invention. Once

formed, an expression cassette comprising an acetyl CoA carboxylase gene can

be subcloned into a known expression vector. The screening method in the

invention includes the steps of introducing an expression vector into a host cell

and detecting and/or quantitating expression of a plant acetyl CoA carboxylase

10   gene. This method of screening is useful to identify expression cassettes

providing for an overexpression of a plant acetyl CoA carboxylase gene,

antisense molecules that effectively inhibit acetyl CoA carboxylase synthesis,

and expression of an acetyl CoA carboxylase in the chloroplast of a transformed

plant cell.

15   Suitable known expression vectors include plasmids that

autonomously replicate in prokaryotic and eukaryotic cells. Specific examples

include plasmids such as the pBI121 or pBI221 plasmid constructed as described

by Jefferson cited supra, a binary Ti vector such as PG582 as described by An

cited supra, PUC119, or PBR322. The preferred expression system is a pBI121

20   or pBI221 plasmid.

An expression cassette of the invention can be subcloned into an

expression vector by standard methods. The expression vector can then be

introduced into prokaryotic or eukaryotic cells by standard methods including

protoplast transformation, silicon fiber-based transformation Agrobacterium

25   mediated transformation, electroporation, microprojectiles, e.g., preferably using

gold particles of about 6 to about 10 microns in diameter although diameters that

are about 100 to about 1000 times smaller may also be employed, and liposomes.

The expression vector can be introduced into plant cells such as tobacco,

Brassica, Black Mexican sweet corn, and Arabidopsis cells. The vector can also

30   be introduced into procaryotic cells such as E. coli or Agrobacterium.

Transformed cells can be selected typically using a selection marker encoded on

the expression vector.

Transient expression of a plant acetyl CoA carboxylase gene can

be detected and quantitated in the transformed cells. Gene expression can be

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quantitated by a quantitative Western blot using antibodies specific for the cloned acetyl CoA carboxylase or by detecting an increase in specific activity of the enzyme. The tissue and subcellular location of the cloned acetyl CoA carboxylase can be determined by immunochemical staining methods using

5   antibodies specific for the cloned acetyl CoA carboxylase. Sensitivity of the cloned acetyl CoA carboxylase to herbicides can also be assessed. Expression cassettes providing for overexpression of a plant acetyl CoA carboxylase or acetyl CoA carboxylase tolerant to herbicides can then be used to transform monocot and/or dicot plant tissue cells and to regenerate transformed plants and

10 seeds.

C.   Method of Imparting Cyclohexanedione or
       Aryloxyphenoxypropanoic Acid Herbicide Tolerance to a Plant

         The invention provides a method of conferring cyclohexanedione

15 or aryloxyphenoxypropanoic acid herbicide tolerance to a plant. The method includes the steps of introducing an expression cassette comprising a gene coding for a plant acetyl CoA carboxylase or a functional mutant thereof operably linked to a promoter into the cells of plant tissue and expressing the gene in an amount effective to render the cells of the plant tissue substantially

20 tolerant to herbicides. An effective amount of gene expression to render the cells of the plant tissue substantially tolerant to the herbicide depends on whether the gene codes for an unaltered acetyl CoA carboxylase gene or a mutant or altered form of the gene that is less sensitive to the herbicides. Expression of an unaltered plant acetyl CoA carboxylase gene in an effective amount is that

25 amount that provides for a 2- to 50-fold increase in herbicide tolerance and preferably increases the amount of acetyl CoA carboxylase from at least about 2- to 20-fold over that amount of the native enzyme. An altered form of the enzyme can be expressed at levels comparable to that of the native enzyme or less if the altered form of the enzyme has higher specific activity. Acetyl CoA

30 carboxylase substantially tolerant to herbicides is an enzyme that is tolerant of levels of herbicide which normally inhibit a native acetyl CoA carboxylase and preferably can function in concentrations of herbicide of about 2- to 20-fold greater than are toxic to the native enzyme.

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Herbicide tolerance can be achieved by at least two methods including: 1) by increasing the level of gene expression of a native or unaltered acetyl CoA carboxylase gene; or 2) by introducing an altered gene coding for an acetyl CoA carboxylase that is less sensitive to herbicide inhibition. The level of
5   gene expression can be increased by either combining a plant acetyl CoA carboxylase gene with a promoter that provides for a high level of gene expression such as the 35S Came promoter or by introducing the gene into the cells so that multiple copies of the gene are integrated into the genome of the transformed plant cell. Formation of an expression cassette comprised of a plant
10   acetyl CoA carboxylase gene operably linked to a promoter that can be expressed in an effective amount to confer herbicide tolerance has been described previously.

Most monocots, but not dicots, are sensitive to cyclohexanedione and/or aryloxyphenoxypropanoic acid herbicides. The preferred plant cells for
15   introducing an expression cassette of the invention to achieve herbicide tolerance for the plant cells then are monocot plants. Monocot plants include corn, wheat, barley, sorghum, rice, and others. An expression cassette of the invention can be introduced by methods of transformation, especially effective for monocots including biolistic transformation of Type II embryogenic suspension cells as
20   described by W.J. Gordon-Kamm et al., Plant Cell, 2, 603-618 (1990), M.E. Fromm et al., Bio/Technology, 8, 833-839 (1990) and D.A. Walters et al., Plant Molecular Biology, 18, 189-200 (1992) or by electroporation of type 1 embryogenic calluses described by D'Hafluin et al., The Plant Cell, 4, 1495 (1992). Transformed cells can be selected for the presence of a selectable
25   marker gene. Transient expression of a plant acetyl CoA carboxylase gene can be detected in the transgenic embryogenic calli using antibodies specific for the cloned plant acetyl CoA carboxylase. Transformed embryogenic calli can be used to generate transgenic plants that exhibit stable inheritance of either the altered acetyl CoA carboxylase gene or overexpression of the acetyl CoA
30   carboxylase gene. Maize cell lines exhibiting satisfactory levels of tolerance to herbicide are put through a plant regeneration protocol to obtain mature maize plants and seeds expressing the tolerance traits such as described in D'Hafluin, cited supra., or An, cited supra. The plant regeneration protocol allows the

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development of somatic embryos and the subsequent growth of roots and shoots. To determine that the herbicide-tolerance trait is expressed in differentiated organs of the plant, and not solely in undifferentiated cell culture, regenerated plants are exposed to herbicide levels which will normally inhibit shoot and root

5   formation and growth.

Mature maize plants are then obtained from maize cell lines that are known to express the trait. If possible, the regenerated plants are self-pollinated. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important inbred lines. Conversely, pollen

10   from plants of these inbred lines is used to pollinate regenerated plants. The genetics of the trait are then characterized by evaluating the segregation of the trait in the first and later generation progeny. Stable inheritance of overexpression of a plant acetyl CoA carboxylase or a functional mutant of a plant acetyl CoA carboxylase conferring herbicide tolerance to the plant is

15   achieved if the plants maintain herbicide tolerance for at least about three to six generations.

Seed from transformed monocot plants regenerated from transformed tissue cultures is grown in the field and self-pollinated to generate true breeding plants. Progenies from these plants become true breeding lines

20   which are evaluated for herbicide tolerance in the field under a range of environmental conditions. Herbicide tolerance must be sufficient to protect the monocot plants at the maximum labeled delivery rate under field conditions which cause herbicides to be most active. Appropriate herbicide concentrations and methods of application are those which are known and have been developed

25   for the cyclohexanedione and/or aryloxyphenoxypropanoic acid herbicides in question.

In a preferred version, an expression cassette comprised of a maize acetyl CoA carboxylase gene isolated from a maize cell line tolerant to sethoxydim and haloxyfop and linked to the 35S Came promoter is introduced

30   into an herbicide sensitive monocot tissue using biolistic transformation. Transformed calli are selected and used to generate transgenic plants. Transformed calli and transgenic plants can be evaluated for tolerance to sethoxydim and haloxyfop and for stable inheritance of the tolerance trait.

D.   Method for Altering the Oil Content in a Plant

The invention also provides a method of altering the oil content in a plant. The method include the steps of introducing an expression cassette comprising a gene coding for plant acetyl CoA carboxylase or functional mutant

5   thereof operably linked to a promoter functional in a plant cell into the cells of plant tissue and expressing the gene in an amount effective to alter the oil content of the plant cell. An alteration in the oil content of a plant cell can include a change in the total oil content over that normally present in that type of plant cell, or a change in the type of oil from that normally present in the plant

10   cell. Expression of the gene in an amount effective to alter the oil content of the gene depends on whether the gene codes for an unaltered acetyl CoA carboxylase or a mutant or altered form of the gene. Expression of an unaltered plant acetyl CoA carboxylase gene in an effective amount is that amount that may provide a change in the oil content of the cell from about 1.2- to 20-fold

15   over that normally present in that plant cell, and preferably increases the amount of acetyl CoA carboxylase about 2- to 20-fold over that amount of the enzyme normally present in that plant cell. An altered form of the enzyme can be expressed at levels comparable to that of the native enzyme or less if the altered form of the enzyme has higher specific activity.

20   An alteration in oil content of the plant cells according to the method of the invention can be achieved in at least two ways including:

(1) an increase or decrease in expression of an unaltered plant acetyl CoA carboxylase gene; or

(2) by introducing an altered or functional mutant plant acetyl

25   CoA carboxylase gene coding for an enzyme that exhibits a change in specific activity.

The level of gene expression of an unaltered plant acetyl CoA carboxylase gene can be increased by either combining an unaltered plant acetyl CoA carboxylase gene with a promoter that provides for a high level of gene expression, such as

30   the 35S cauliflower mosaic virus or by introducing the expression cassette and/or selecting for plant cells having multiple copies of a plant acetyl CoA carboxylase gene integrated into the genome. A decrease in expression of an unaltered acetyl CoA carboxylase can be achieved by transformation with an ACCase antisense

gene containing an expression cassette. When an altered or functional mutant plant acetyl CoA carboxylase gene codes for an enzyme that has an increase in specific activity, it may lead to an increase in total oil content of a plant cell even if the level of gene expression is comparable to that of the native enzyme. When
5   an altered or functional mutant acetyl CoA carboxylase gene codes for an enzyme having a decrease in specific activity, it may lead to a decrease in the total oil content of the plant cell compared to that normally present.

An expression cassette as described above can be introduced into either monocots or dicots. Preferably, the expression cassette is introduced into
10   dicot plants such as soybean, canola, and sunflower. An expression cassette can be introduced by standard methods including protoplast transformation, Agrobacterium-mediated transformation, microprojectiles, electroporation, and the like. Transformed cells or tissues can be selected for the presence of a selectable marker gene.

15   Transient expression of a plant acetyl CoA carboxylase gene can be detected in transformed cells or tissues by immunoreactivity with antibodies specific for the cloned acetyl CoA carboxylase. Overexpression of a plant acetyl CoA carboxylase can be detected by quantitative Western blots. A change in specific activity of the enzyme can be detected by measuring enzyme activity in
20   the transformed cells. A change in total oil content can also be examined by standard methods, as described in Clark & Snyder, JACS, 66:1316 (1989).

Transgenic plants and seeds can be generated from transformed cells and tissues showing a change in oil content or in the amount or specific activity of a plant acetyl CoA carboxylase using standard methods. It is
25   especially preferred that the oil content of the leaves, seeds, or fruits is increased.

In a preferred version, a maize acetyl CoA carboxylase gene is combined with a 35S cauliflower mosaic virus promoter in a vector such as pBI121 or pBI221 and introduced into soybean cells using the microprojectile method. Transformed soybean cells showing an increase in expression of acetyl
30   CoA carboxylase of at least about 2-fold or at least a 1.2-fold increase in oil content are selected. Transformed soybean cells exhibiting overexpression of acetyl CoA carboxylase or showing an increase in total oil content are used to generate transgenic plants and seeds.

30

E.   Method of Producing Plant Acetyl CoA Carboxylase

The invention also provides a method of producing plant acetyl CoA carboxylase in a host cell. The method includes the steps of introducing an expression cassette comprised of a gene encoding a plant acetyl CoA

5   carboxylase or functional mutant thereof into a host cell and expressing the gene in an amount sufficient to allow for crystallization of the plant acetyl CoA carboxylase. An amount sufficient to allow for crystallization of a plant acetyl CoA carboxylase is about 20- to 100-fold increase over the amount of plant acetyl CoA carboxylase that can normally be purified from plant cells, preferably

10   about 2 to 10 mg protein. Crystallized plant acetyl CoA carboxylase can be used to identify other herbicides that can bind to and inhibit acetyl CoA carboxylase function. In addition, the availability of large amounts of purified enzyme provides for screening of the efficacy of such herbicides.

An expression cassette can include a promoter that is functional in

15   either a eukaryotic or prokaryotic cell. The expression cassette can be introduced into a prokaryotic cell such as E. coli, or a eukaryotic cell such as a plant or yeast. The preferred cell is a prokaryotic cell used routinely in producing recombinant proteins such as E. coli. The expression cassette can be introduced and transformed cells selected by standard methods.

20   The plant acetyl CoA carboxylase gene can be expressed in an prokaryotic cell until sufficient amount of the enzyme is produced so that it can be crystallized. Plant acetyl CoA carboxylase can be isolated from bacterial cells using standard methods, including those described in Example V. The purified acetyl CoA carboxylase can then be crystallized and characterized by standard

25   methods.

EXAMPLE I

Identification of Herbicide Mechanism and Site of Action

The objective of this Example was to identify the mechanism

30   whereby sethoxydim and/or haloxyfop inhibit fatty acid synthesis in maize. The results, reported in J. D. Burton et al., Biochem. Biophys. Res. Comm., 148, 1039 (November 13, 1987), show that both sethoxydim and haloxyfop inhibit acetyl-coenzyme A carboxylase (ACCase) (EC 6.4.1.2) in maize chloroplasts.

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A.   **Chemicals**

Buffers and cofactors were purchased from Sigma Chemical
Company (St. Louis, Missouri); [2-^{14}C]acetate was purchased from Research
Products International; [2-^{14}C]pyruvate and [^{14}C]NaHCO$_3$ were purchased from
5   New England Nuclear; and [2-^{14}C]malonyl coenzyme A was purchased from
Amersham. Sethoxydim was a gift from BASF (Parsippany, New Jersey), and
haloxyfop was provided by Dow Chemical USA (Midland, Michigan).

B.   **Plant Growth Conditions**

Corn (*Z. mays L.*, 'B37 x Oh43') seeds were germinated in
10   darkness for 96 hours in vermiculite in an incubation chamber maintained at
30°C, 80% RH. Seedlings were then transferred to a growth chamber with a 16
hour light (25°C) and an 8 hour dark (20°C) cycle, 90% relative humidity (RH).
After greening 48 hours, seedlings were returned to the dark incubation chamber
for 12 hours to deplete chloroplast starch reserves. Seedlings were harvested 6
15   days after planting. Pea (*P. sativum L.*, 'PI 9901-C') seedlings were grown in
vermiculite in a growth chamber with a 16 hour light (21°C) and 8 hour dark
(16°C) cycle, 80% RH. Peas were harvested 10 to 13 days after planting. Black
Mexican Sweet (BMS) corn suspension cultures were maintained in a
supplemented Murashige-Skoog (MS) medium (C. E. Green, *Hort. Sci.*, 12, 7-10
20   (1977)), and subcultured weekly by 20-fold dilution of the suspension culture
into fresh medium.

C.   **Chloroplast Isolation**

Chloroplasts from corn and pea seedlings were isolated at 4°C (K.
Cline et al., *J. Biol. Chem.*, 260, 3691-3696 (1985)). Seedlings (50 g of shoots)
25   were homogenized in 200 ml buffer A (50 mM HEPES-NaOH pH 7.5, 330 mM
sorbitol, 0.1% w/v BSA, 1 mM MgCl$_2$, 1 mM MnCl$_2$, 2 mM EDTA, 5 mM
isoascorbate, 1.3 mM glutathione) in an omnimixer (five, 3-second bursts at full
speed). The homogenate was filtered through six layers of cheesecloth and two
layers of miracloth, and then centrifuged at 3000 g for 3 minutes with hand-
30   braking. The pellet was gently resuspended in buffer A and layered onto a
preformed linear Percoll gradient (50 mM HEPES-NaOH pH 7.5, 330 mM
sorbitol, 1.9 mM isoascorbate, 1.08 mM glutathione, 0.1% w/v BSA, 50%
Percoll) which was centrifuged at 3000 g for 20 minutes in a Sorvall HB-4 rotor.

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The lower band in the gradient, containing intact chloroplasts, was washed twice
by gently resuspending it in 20 ml of buffer B (50 mM HEPES-NaOH, pH 7.5,
and 330 mM sorbitol) followed by repelleting (3000 g, 5 minutes). The final
pellet, consisting of intact chloroplasts, was resuspended in 2 to 3 ml of buffer B
5   and stored on ice in the dark until use.

D.   **Fatty Acid Synthesis**

[^{14}C]acetate and [^{14}C]pyruvate were used as precursors to measure
fatty acid biosynthesis in isolated chloroplasts (B. Liedvogel et al., Planta, 169,
481-489 (1986)). [^{14}C]acetate incorporation was assayed in a 0.5 ml-volume
10   containing: 50 mM HEPES-NaOH (pH 7.5), 330 mM sorbitol, 5 mM KH_2PO_4,
10 mM $NaHCO_3$, 1 mM $MgCl_2$, 1 mM ATP, 0.1 mM CoA, 0.15 mM
[^{14}C]acetate (3.33 mCi/mmol), and chloroplasts (20 to 50 µg chlorophyll).
[^{14}C]pyruvate incorporation into fatty acids was assayed in the same medium
except that it included 2 mM TPP, 1 mM NAD^+, 0.15 mM [^{14}C]-pyruvate (1.33
15   mCi/mmol), but no acetate. Assay suspensions were illuminated with 1400
µE/m^2·second PAR at 25°C. Assays were initiated by the addition of the labeled
substrate and stopped by the addition of 0.5 ml of 40% KOH. To determine the
incorporation of radiolabel into a non-polar (fatty acid) fraction, each treatment
was saponified at 90°C for 30 minutes in capped vials (P. B. Hoj et al., Carlsberg
20   Res. Commun., 47, 119-141 (1982)). The vials were acidified with 0.5 ml 40%
H_2SO_4, and carrier fatty acids (20 µg each of C 14:0, C 16:0, and C 18:0) were
added. The assay mixture was extracted twice with 4 ml hexane. The extracts
were combined, dried under N_2, and redissolved in 0.3 ml hexane. Aliquots (50
µl) were counted for radioactivity by liquid scintillation spectrometry.

25   Incorporation of [^{14}C]malonyl-Coenzyme A into fatty acids (P. B.
Hoj et al., supra; and J. B. Ohlrogge et al., Proc. Natl. Acad. Sci. USA, 76, 1194-
1198 (1979)) was assayed using cell-free preparations from BMS tissue culture.
Cells harvested during logarithmic growth phase were frozen in liquid nitrogen,
ground with a mortar and pestle, and thawed in a medium containing: 0.1 M
30   HEPES-KOH, pH 7.5; 0.3 M glycerol, and 5 mM DTT (buffer:tissue, 2:1, v/w).
The homogenate was centrifuged at 12,000 g for 20 minutes. The supernatant
was filtered through miracloth and centrifuged (125,000 g) for 60 minutes and
then filtered through miracloth and assayed. Assays were conducted at 25°C in a

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0.4 ml volume containing: 1.0 mM ATP, 0.32 mM NADPH, 0.38 mM NADH, 25 μM CoA, 10 μM acetyl-CoA, 25 μg acyl-carrier protein, and 12 μM malonyl-CoA (11.54 μCi/μmol). Reactions were initiated by addition of [^{14}C]malonyl CoA and stopped by addition of 0.4 ml 40% KOH. Label incorporation into

5   fatty acids was determined as above. Chlorophyll (D. I. Arnon, Plant Physiol., 24, 1-15 (1949)) and protein (P. K. Smith et al., Anal. Biochem., 150, 76-85 (1985)) were determined as described therein.

E.   Acetyl-Coenzyme A Carboxylase (ACCase) Activity

Maize chloroplasts, isolated as described above, were suspended

10   in buffer C (0.1 M Tricine-KOH, pH 8.0; 0.3 M glycerol, and 1 mM DTT) and homogenized in a glass tissue homogenizer. The disrupted chloroplast fraction was centrifuged at 16,000 g for 15 minutes. The supernatant was desalted on a Sephadex G-25 column (1.5 x 5 cm equilibrated with 0.1 M Tricine-KOH, pH 8.0; and 0.3 M glycerol) and assayed directly. ACCase activity (B. J. Nikolau et

15   al., Arch. Biochem. Biophys., 211, 605-612 (1981)) was assayed at 30°C in a 0.2 ml volume which contained 1 mM ATP, 3 mM acetyl coenzyme A, 2.5 mM MgCl$_2$, 50 mM KCl, 0.5 mM DTT, and 15 mM [^{14}C]NaHCO$_3$ (0.17 mCi/mmol). Reactions were initiated by addition of acetyl coenzyme A and stopped by addition of 25 μl of 12 N HCl. Product formation was determined by the

20   radioactivity found in an acid stable fraction by liquid scintillation spectrometry. Enzyme activity was linear for 15 minutes.

F.   Results

To probe for the site of herbicidal activity of sethoxydim and haloxyfop, labeled acetate, pyruvate, and malonyl-CoA were used individually

25   as precursors for fatty acid synthesis. Isolated chloroplasts from corn seedlings incorporated [^{14}C]acetate and [^{14}C]pyruvate into a non-polar fraction (fatty acids). Acetate incorporation was linear for 30 min after a 5 min lag period, and dependent upon the addition of free acetyl coenzyme A. Addition of either 10 μM sethoxydim or 1 μM haloxyfop inhibited {^{14}C]acetate incorporation into fatty

30   acids by 90% and 89%, respectively, as shown in Table I, below. Sethoxydim (10 μM) and haloxyfop (1 μM) also inhibited the incorporation of [^{14}C]pyruvate into fatty acids by 98% and 99%, respectively.

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TABLE I

Inhibition of [14C]acetate and [14C]pyruvate
Incorporation into Fatty Acids in
Corn Seedling Chloroplasts by
Sethoxydim (10 μM) and Haloxyfop (1 μM),
10 minute assay time

	Acetate	Pyruvate
	---Activity (nmol/mg chl·min)---	
Control	4.4 ± 0.4^1	10.8 ± 2.3
	--------------% Inhibition----------------	
Sethoxydim	90 ± 2.5	98 ± 1.1
Haloxyfop	89 ± 3.1	99 ± 0.3

[1]Results are expressed as mean of two experiments ± standard error.

The effect of 10 μM sethoxydim and 1 μM haloxyfop on [14C]malonyl-CoA incorporation into fatty acids was determined using cell-free extracts from corn suspension cultures. Neither sethoxydim (10 μM) nor haloxyfop (1 μM) inhibited fatty acid synthetase activity. Thus, both herbicides inhibited fatty acid synthesis in intact chloroplasts from corn seedlings with either acetate or pyruvate as a precursor, but did not inhibit incorporation of malonyl-CoA into fatty acids. This suggests that ACCase which catalyzes the formation of malonyl-CoA is the site of action of these herbicides.

EXAMPLE II

Selection and Characterization of Herbicide-tolerant Cell Lines

A selection protocol to identify and isolate herbicide-tolerant maize cells was developed to minimize the adverse effects of high herbicide concentrations on somatic embryo development and plant regeneration capacity. The procedure involved exposing tissue to gradually increasing concentrations of herbicide beginning with a sethoxydim concentration representing 1/20th of lethal dose and doubling the herbicide concentration at approximately two-week intervals until the lethal dose (10 μM sethoxydim) was reached. In this way, the

35

herbicide was allowed to take effect slowly with continuous selection pressure, thus permitting herbicide-tolerant cells to accumulate over time while not affecting the potential for plant regeneration.

A.   Selection of a Sethoxydim-Tolerant Cell Line

5   Many selections were carried out utilizing the selection protocol described in the preceding paragraph. The selection of one such sethoxydim-tolerant cell line that was identified and characterized is described below in detail.

Approximately 100 grams of vigorously growing, regenerable,

10   friable, embryogenic maize callus tissue established from an F_1 immature embryo resulting from the cross A188 x B73 were transferred to agar-solidified maintenance medium (Armstrong and Green, Planta, 164, 207 (1985)) in petri plates containing 0.5 μM sethoxydim (BASF) (Parsippany, New Jersey). This callus line was designated 2167-9/2160-154. Forty plates were prepared and five

15   clumps of callus tissue weighing about 0.5 grams each were placed on each plate. The 0.5 μM sethoxydim concentration was chosen from growth inhibition studies to provide less than 10-20% growth inhibition during the first two weeks of herbicide exposure. After 14 days, 0.25-0.5 gram pieces of tissue showing vigorous growth rate and retention of embryogenic morphology (i.e., presence of

20   somatic embryos) were subcultured on fresh medium containing 1.0 μM sethoxydim. Eighty plates containing five pieces of tissue per plate were prepared. For each subsequent transfer, all callus tissue showing growth and somatic embryo forming ability was placed on fresh media containing a two-fold increased sethoxydim concentration. Therefore, callus was transferred at two-

25   week intervals to petri plates containing 0.5, 1.0, 2.0, 5.0 and 10.0 μM sethoxydim. During the course of the selection process, the total number of lines decreased as the herbicide-mediated growth inhibition became more intense. Cell lines exhibiting growth on 10 μM sethoxydim were designated as herbicide-tolerant and given an identification number. Two sethoxydim-tolerant lines were

30   recovered that exhibited uninhibited growth at 10 μM sethoxydim. These lines were designated 2167-9/2160-154 S-1 and 2167-9/2160-154 S-2.

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B.   **Characterization of Herbicide-Tolerant Maize Cell Line
        2167-9/2160-154 S-2**

                Tolerant cell line 2167-9/2160-154 S-2 ("S-2") was characterized

to evaluate: (1) the magnitude of sethoxydim tolerance; (2) cross-tolerance of

5   haloxyfop; and (3) the biochemical basis for the tolerance. Callus tissue from S-

2 that had been maintained on 10 µM sethoxydim was transferred to media

containing up to 100 µM sethoxydim. One-half gram of S-2 tissue was plated on

a 7 cm filter paper as a lawn overlaying 50 ml agar-solidified culture medium

containing 0, 0.5, 1.0, 2.0, 5.0, 10.0, 50.0 and 100 µM sethoxydim, and cultured

10   for two weeks. Control cell line 2167-9/2160-154 was plated similarly on

medium containing the same levels of sethoxydim. The results of this study are

summarized in Figure 2. The control cell line growth after two weeks was

inhibited 50% at 1 µM sethoxydim. Growth of S-2 was not inhibited at 100 µM

sethoxydim, indicating that S-2 was at least 100-fold more tolerant than the

15   control callus line.

                Growth of S-2 was inhibited with 0.65 µM haloxyfop, whereas

the control cell line was inhibited 50% with 0.02 µM, indicating approximately a

30-fold increase in tolerance.

C.   **Acetyl-Coenzyme A Carboxylase (ACCase) Activity of Maize Cell**
20   **Line S-2**

                Assays were conducted to determine if ACCase extracted from

cell line S-2 was altered with respect to herbicide activity. ACCase activity of

control tissue was 50% inhibited either by 1.5 µM sethoxydim, or by 0.25 µM

haloxyfop. ACCase activity of S-2 tissue was inhibited 50% either by 70 µM

25   sethoxydim, or by 1.8 µM haloxyfop, indicating at least 40-fold and 7-fold

decreases in herbicide sensitivity on concentration basis, respectively.

EXAMPLE III

Plant Regeneration and Production of Herbicide-Tolerant Seed

A.   **Plant Regeneration Protocol**

30   Sixteen ca. 150 mg clumps of S-2 callus were transferred per 25 x

100 mm petri plate containing agar-solidified N6 basal salts and 6% sucrose and

incubated 7-14 days in low light (20 µE m^{-2} s^{-1}). Several plates containing callus

on plant regeneration medium were prepared. Callus was transferred to agar-

solidified Murashige-Skoog (MS) medium without hormones and incubated in

high intensity light (200 μE m-2 s⁻¹) for shoot elongation. Developing plants (1-

3 cm long) were isolated from the callus surface and transferred to magenta

boxes containing agar-solidified MS salts, 2% sucrose with no hormones for two

5   weeks of further growth. When plants reached the 2-3 leaf stage, they were

transplanted to peat pots containing potting soil, and were incubated in the

growth room until growing stably. Surviving plants were transferred to soil in 4"

diameter plastic pots and grown in the greenhouse.

B.   Expression of Herbicide Tolerance in Plants Regenerated from S-2
10   **Callus Tissue**

Groups of eight control (2167-9/2160-154 unselected) and eight

S-2 plants were sprayed with either 0.0, 0.01, 0.05, 0.11, 0.22 or 0.44 kg/ha

sethoxydim to determine whole plant sethoxydim-tolerance of greenhouse-

grown plants. Control plants were killed by 0.05 kg/ha or more sethoxydim.

15   Plants regenerated from the S-2 cell line survived the 0.44 kg/ha sethoxydim

treatment, indicating that S-2 plants exhibit at least 20-fold more tolerance of

sethoxydim than control. Figure 3 shows the growth response of the regenerated

plants seven days after treatment with 0.44 kg/ha sethoxydim. As shown in

Figure 4, shoot height of regenerated S-2 plants was only slightly reduced 14

20   days after treatment with 0.44 kg/ha sethoxydim.

C.   Seed Production from S-2 Plants

Plants surviving sethoxydim treatments of up to 0.44 kg/ha were

transplanted to the genetics plot on the University of Minnesota campus, St.

Paul, Minnesota. Additional S-2 plants were transplanted to the field that had

25   not been sprayed. Sixty-five 2167-9/2160-154 control plants and ninety-five S-2

plants were grown to maturity in the field. Plants were either self-pollinated or

cross-pollinated to inbred maize lines A188, A619, A641, A661, A665, B37,

B73, R806, and W153R. Control seed were produced by selfing 2167-9/2160-

154 regenerated plants, or by crossing them with the inbreds listed above.

30 **D.   Expression of Herbicide Tolerance in Progeny of Regenerated Plants**

Seeds obtained by the crossing procedure described above were

viable and germinated normally. Seeds from thirty S-2 selfed plants and fifteen

2167-9/2160-154 control plants were planted in 25 x 50 cm trays of soil (28

seeds from each plant in one tray) and grown in the greenhouse. Seedlings at the
3-4 leaf stage were treated with 0.1, 0.44, and 1.1 kg/ha sethoxydim and
evaluated for visual herbicide damage and shoot height. Based on visual rating
of herbicide damage two weeks after treatment, selfed progeny of S-2 plants

5   segregated approximately 1:2:1 for healthy, uninjured plants: to plants showing
partial injury: to dead plants, respectively, at 0.44 and 1.1 kg/ha sethoxydim
treatments. All control progeny of 2167-9/2160-154 control plants were killed
by 0.1 kg/ha and greater levels of sethoxydim. These results demonstrate
dominant expression of sethoxydim tolerance indicating that sethoxydim

10   tolerance in S-2 plants is a heritable trait. Similar tests were conducted on
progeny of S-2 plants crossed to the other inbreds. In all cases, these test cross
progeny treated with 0.44 kg/ha sethoxydim segregated 1:1 for growing shoots
versus dead shoots whether S-2 plants were used as male or female parents.
These results confirm that sethoxydim tolerance is controlled by a single

15   dominant nuclear gene. In all cases, control plants crossed to the other inbreds
were killed and therefore sethoxydim-sensitive.

E.   **Method for Obtaining Uniform Herbicide-Tolerant Seed**

Progeny of S-2 plants surviving sethoxydim treatments of 0.44
and 1.1 kg/ha and showing no herbicide injury were transferred to the

20   greenhouse and grown to maturity. These plants may be selfed and their
progeny evaluated for sethoxydim and haloxyfop tolerance to identify pure
breeding herbicide-tolerant maize lines.

Progeny of S-2 plants crossed to inbred lines and exhibiting
sethoxydim tolerance may be recurrently backcrossed to the same inbreds.

25   Progeny of each cross may be screened for sethoxydim-tolerance, and tolerant
plants grown to maturity and again crossed to the recurrent parent. After six or
seven cycles of backcrossing, sethoxydim-tolerant plants may be selfed and
progeny screened for tolerance to produce homozygous sethoxydim tolerant
maize inbreds.

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EXAMPLE IV

Selection of Additional Herbicide-Tolerant Maize Cell Lines

One primarily sethoxydim-tolerant maize cell line, 2167-9/2160-

5 154 S-1, and two haloxyfop-tolerant maize cell lines, 2167-9/2160-154 H-1 and

2167-9/2160-154 H-2, were selected and characterized as follows:

A.   **Selection of Maize Cell Line 2167-9/2160-154 S-1**

Maize cell line 2167-9/2160-154 S-1 was selected from maize

cell culture using the protocol described in detail above for the selection of Line

10 2167-9/2160-154 S-2. Approximately 70 plants were regenerated from Line

2167-9/2160-154 S-1, and either self-pollinated or cross-pollinated to the inbred

maize lines A188, A619, A641, A661, A665, B37, B73, R806, and W153R.

B.   **Selection of Maize Cell Line 2167-9/2160-154 H-1**

Line 2167-9/2160-154 H-1 was selected from maize cell culture

15 using a similar protocol described in detail above except maize callus tissue was

selected using the herbicide haloxyfop. Maize callus tissue was initially plated

on 0.01 μM haloxyfop. At two-week intervals, surviving tissue was subcultured

onto 0.05, 0.10 and 0.20 μM haloxyfop. Approximately 50 plants were

regenerated from Line 2167-9/2160-154 H-1, and were self-pollinated.

20 C.   **Selection of Maize Cell Line 2167-9/2160-154 H-2**

Line 2167-9/2160-154 H-2 was selected from maize cell culture

using a similar protocol described in detail for line 2167-9/2160-154 H-1. No

plants have been successfully regenerated from this line.

D.   **Characterization of Lines 2167-9/2160-154 S-1, H-1 and H-2**

25 The tolerant callus cultures were characterized to determine the

magnitude of sethoxydim and haloxyfop tolerance. Callus tissue from these

lines was evaluated in experiments as described above in the characterization of

line 2167-9/2160-154 S-2. Table II summarizes the results of these studies.

Line 2167-9/2160-154 S-1 and Line 2167-9/2160-154 H-2 showed a four-fold

30 increase in haloxyfop tolerance, while Line 2167-9/2160-154 H-1 exhibited

approximately a 60-fold increase in haloxyfop tolerance. Neither haloxyfop

selected line showed a significant degree of sethoxydim tolerance, while the

40

sethoxydim selected line S-1 exhibited approximately a 100-fold increase in
sethoxydim tolerance.

TABLE II

Herbicide Tolerance of Cell Lines S-1, H-1 and H-2

Cell Line	Herbicide	
	Haloxyfop	Sethoxydim
2167-9/2160-154 S-1	4^1	100
2167-9/2160-154 H-1	61	0
2167-9/2160-154 H-2	4	0

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[1]The numbers represent the fold increase in herbicide concentration that results
in a 50% reduction in growth of the selected cell lines compared to the
unselected control cell line 2167-9/2160-154.

E.   **Herbicide Inhibition of Acetyl Coenzyme A Carboxylase of Maize
         Cell Lines S-1, H-1 and H-2**

Acetyl Coenzyme A Carboxylase (ACCase) was extracted from
cell lines S-1, H-1 and H-2 and assayed as described in detail for maize cell line
S-2, above. Table III below summarizes the results of these studies. The
ACCase from line S-1 was more tolerant of both sethoxydim and haloxyfop,
while the ACCase from line H-1 was more tolerant of haloxyfop, but not of
sethoxydim. The ACCase from line H-2 showed no difference from the
unselected parent line 2167-9/2160-154 in sensitivity to either herbicide.

However, cell line H-2 exhibited approximately a five-fold higher
level of ACCase activity as compared to the unselected parent line 2167-9/2160-
154. Thus, selection for sethoxydim or haloxyfop tolerance resulted in a less

sensitive ACCase in cell line S-1 and H-1, as well as a higher level of ACCase
activity in cell line H-2.

TABLE III

Herbicide Inhibition of ACCase of Maize
Cell Lines S-1, H-1 and H-2

	Herbicide	
Cell Line	Haloxyfop	Sethoxydim
2167-9/2160-154 S-1	3	4
2167-9/2160-154 H-1	7	0
2167-9/2160-154 H-2	0	0

[1]The numbers represent the fold increase in herbicide concentration that inhibits
ACCase activity of the selected cell lines by 50% compared to the unselected
parent cell line 2167-9/2160-154.

Deposit of Seeds

Seeds from representative S-2 plants (Ex. III (B)) and H-1 plants
(Ex. IV(B)) have been deposited with the American Type Culture Collection,
12301 Parklawn Drive, Rockville, MD 20852 USA on October 25, 1988 and
assigned accession numbers ATCC 40507, and ATCC 40508, respectively.

EXAMPLE V

Formation of cDNA Clones Encoding ACCase

A.   ACCase Purification

The acetyl CoA carboxylase enzyme was isolated and purified
from plant tissues and characterized. The purified enzyme was used to generate
antibody reagents useful in identifying cDNA clones encoding the gene or
portions of the gene for ACCase.

ACCase was extracted from frozen shoots of 7-d-old maize (Zea
Mays L. inbred A619 or B73) seedlings grown in a growth chamber (24°C, 90%
RH, 16-h daylength at 210 μE m^{-2} s^{-1}). The outermost leaf and blade were
removed and the remainder of the shoot was frozen in liquid N_2. Embryos and
endosperm tissue from developing kernels were harvested from field-grown ears
at 36 to 40 days after pollination (DAP). Black Mexican Sweet corn (BMS)

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maize suspension cells were obtained from cultures as previously described (W.B. Parker et al., Plant Physiol., 92, 1220-1225 (1990)). Tissues were stored in liquid N₂ until used.

Extraction and purification steps were performed at 0 to 4°C.

5   Crude extracts of leaf, bundle sheath strands, embryo, endosperm, and BMS cells were prepared from frozen tissue as described by W.B. Parker et al., Proc. Nat'l Acad. Sci. USA, 87, 7175-7179 (1990), except that extraction buffer contained 0.1 M Tricine-KOH, pH 8.3, 0.3 M glycerol, 5 mM DTT, 2mM Na₂EDTA, and 0.5 mM phenyl methonyl sulfonyl fluoride (PMSF). Triton X-

10   100 (0.01% v/v) was added to bundle sheath strand extracts and to some whole leaf extracts. For some experiments, additional protease inhibitors (leupeptin, 2 µg mL; pepstatin A, 100 µg mL⁻¹; benzamidine, 1 mM; ε-amino-n-caproic acid, 5 mM; and soybean trypsin inhibitor, 10 µg mL⁻¹) were included. Filtered homogenates were centrifuged 20 minutes at 30,000g. A portion of the crude

15   supernatant fraction was immediately boiled 5 minutes in 1 volume of SDS sample buffer (W.B. Parker et al., Plant Physiol., 92, 1220-1225 (1990)) for SDS-PAGE analysis; the remainder was desalted on a 10-mL Sephadex G-25 column into extraction buffer minus PMSF.

ACCase was purified from the crude extract supernatant in four

20   steps. This fraction was brought to 30% saturation with solid (NH₄)₂SO₄, stirred 15 minutes, and centrifuged 20 minute at 20,000g. The supernatant was then brought to 40% saturation with (NH₄)₂SO₄ solution, stirred 30 minutes, and centrifuged. The pellet was dissolved in 5·mL extraction buffer, microfuged 5 minutes, and the resulting supernatant was applied to a Sephacryl S-300 gel

25   filtration column (Pharmacia; 2.5 x 46 cm) equilibrated with S-300 buffer (0.1 M Tricine-KOH, pH 8.3, 0.5 M glycerol, 0.5 mM DTT, 2 mM Na50 mM KCl). In later experiments a Sephacryl S-400 column was used. Fractions (2.5 mL) were eluted at 0.75 mL min⁻¹. ACCase activity eluted shortly after the void A₂₈₀ peak (V₀ = 75 mL). Active fractions were pooled, brought to 4.25 mM MgCl₂ (from a

30   0.5 M solution), and applied at 0.2 mL min⁻¹ to a Blue Sepharose CL-6B (Pharmacia; 1.5 x 15 cm) equilibrated with Blue sepharose buffer (S-300 buffer containing 4.25 mM MgCl₂ and 10 mM NaHCO₃). The column was washed overnight with 150 mL buffer (0.45 mL min⁻¹). ACCase activity was then eluted

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with 50 mL buffer plus 10 mM ATP (0.45 mL min^{-1}). Active fractions were

pooled and applied to an FPLC Mono-Q HR 5/5 anion-exchange column

(Pharmacia) equilibrated with S-300 buffer minus KCl. The column was washed

with 30 mL S-300 buffer minus KCl and then with a 48-mL, 0 to 500 mM KCl

5   gradient in S-300 buffer (0.25 mL min^{-1}). Fractions (1 mL) from the two peaks

of ACCase activity were pooled separately. All purification fractions were

desalted into S-300 buffer and assayed for ACCase activity and protein.

ACCase was also analyzed from mesophyll chloroplasts and

bundle sheath strands. Mesophyll chloroplasts from homogenates of 7- to 8-day-

10 old seedlings that were kept in the dark 24 hours prior to harvesting were

isolated on a linear Percoll gradient according to J.D. Burton et al., Pesticide

Biochemistry and Physiology, 34, 76-85 (1989), except that buffers contained

0.6 M sorbitol and centrifugation g-forces were reduced by 25%. Intact

chloroplasts were taken from the discrete lower green band present after Percoll

15 gradient centrifugation (G. Morioux et al., Plant Physiol., 67, 470-473 (1981)).

Pelleted chloroplasts were lysed by resuspending them in ACCase extraction

buffer plus PMSF and 0.01% (v/v) Triton X-100. Bundle sheath strands were

obtained from the original leaf homogenate material retained on a 70-μm filter

after re-homogenizing the retentate five times in a total of 2 L buffer. Triton

20 X-treated, desalted leaf, mesophyll chloroplast, and bundle sheath strand extracts

were assayed for activities of Rubisco (G. Zhu et al., Plant Physiol., 97, 1348-

1353 (1991)), NADP-dependent malate dehydrogenase (M.D. Hatch et al.,

Biochem. Biophys. Res. Commun., 34, 589-593 (1969)), phosphoenolpyruvate

carboxylase (R.C. Leegood et al., "Isolation of Membranes and Organelles from

25 Plant Cells," Academic Press, New York, 185-210(1983)), catalase

(Worthington Biochemicals, 1972), and fumarase (R.L. Hill et al., Methods

Enzymol., 13, 91-99 (1969)), and for total chlorophyll (D.E. Arnon, Plant

Physiol., 24, 1-5 (1949)). Mesophyll chloroplast preparations were judged to be

relatively free of contamination by bundle sheath chloroplasts because they

30 contained 3-fold greater NADP-dependent malate dehydrogenase and one-tenth

as much Rubisco activity (mg^{-1} chlorophyll) than bundle sheath strand extracts.

Mesophyll chloroplast preparations also contained ≤2.6% as much catalase,

fumarase, and phosphoenolpyruvate carboxylase activities (mg^{-1} chlorophyll) as

44

did whole-leaf extracts, indicating they were relatively free of peroxisomal, mitochondrial, or cytoplasmic components.

ACCase activity as measured by acetyl-CoA-dependent $H^{14}CO_3^-$ (ICN, 2.07 GBq mmol^{-1}) incorporation into acid-stable product previously shown

5   to be malonyl-CoA (J.D. Burton et al., _Pesticide Biochemistry and Physiology_, _34_, 76-85 (1989)). Assays of desalted purification fractions or crude, desalted tissue extracts contained up to 50 and 25% (v/v) enzyme, respectively. In some experiments methylcrotonyl-CoA or propionyl-CoA were substituted for acetyl-CoA (E.S. Wurtele et al., _Archives of Biochemistry and Biophysics_, _278_,

10 179-186 (1990)). Avidin (10 U mL^{-1}) was included in some assays. Herbicide inhibition assays contained 1% (v/v) ethanol plus or minus 1 μM haloxyfop (2-[4-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy] propanoic acid, Dow Chemical Co. analytical grade racemic mixture) or 10 μM sethoxydim (2-[1[(ethoxylmino)butyl]-5-[2-(ethylthio)-propyl]-3-hydroxy-2-cyclohexene -1 -

15 one, Li salt, BASF Corp. technical grade). Data are means plus standard error of three assays.

Protein concentrations were determined in duplicate with the Bio-Rad Coomassie blue dye-binding assay as described by the manufacturer, using BSA as the standard.

20   Centrifuged crude extracts and proteins in purification fractions and immunoprecipitation supernatants were separated by SDS-PAGE in 6 or 7.5% gels as previously described (W.B. Parker et al., _Plant Physiol._, _92_, 1220-1225 (1990)). Purification fractions were precipitated in 10% (v/v) TCA, washed with 80% (v/v) acetone, and air-dried 10 minutes prior to

25 electrophoresis. Proteins in gels were stained with silver (J. Heukeshoven et al., _Electrophoresis_, _6_, 103-112 (1985)). High molecular weight protein standards for SDS-PAGE (Pharmacia) were used to estimate polypeptide masses.

The four-step purification procedure shown in Table IV typically yielded 30 to 190 μg of highly purified ACCase from 50 grams (fresh weight) of

30 maize inbred A619 or B73 seedling leaves. ACCase activity in the crude supernatant fraction precipitated between 30 and 40% saturation with $(NH_4)_2SO_4$, which appeared to increase total ACCase activity approximately 38%. Crude extract components might have depressed the reaction rate shown in Table IV

45

because the assay mixture contained 50% enzyme (v/v). In tests of fractions
from another purification, enzyme velocity was proportional to enzyme
concentration in assay mixtures containing up to 25% (v/v) crude extract, but
50% (v/v) mixtures were not tested. ACCase activity eluted from the Sephacryl
5   S-300 gel filtration column slightly after the green void peak. Approximately
56% of the S-300 fraction ACCase activity was recovered from the Blue
Sepharose column, primarily in the initial ATP-containing fractions (12.5 mL).
Both 10 mM NaHCO$_3$ and 4.25 mM MgCl$_2$ (1- and 0.85-fold standard assay
concentrations, respectively) were included in the Blue Sepharose buffer because
10   they improved the total and specific ACCase activity remaining after batch
absorption to Blue Sepharose beads, elution with ATP, and desalting into
extraction buffer minus PMSF. Neither NaHCO$_3$ nor MgCl$_2$ improved enzyme
stability of crude extracts. Mono-Q anion-exchange chromatography resulted in
separation of two ACCase activity peaks which eluted at approximately 210 mM
15   (designated ACCase II) and 250 mM KCl (designated ACCase I), as previously
observed for a hybrid maize variety (J.L. Howard et al., FEBS Lett., 261,
261-264 (1990)). ACCase I comprised about 85% of the total activity recovered
from the column (29% of the original crude extract activity) and had high
specific activity (Table IV). The specific activity of ACCase II was less than
20   30% that of ACCase I. Both activities were inhibited >90% by avidin, as
previously reported (J.L. Howard et al., FEBS Lett., 261, 261-264 (1990)). The
mass of native ACCase I was estimated to be approximately 490 kD by gel
filtration on Superose 6.

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TABLE IV

Purification of ACCase I From
Maize Inbred A619 Seedling Leaves[a]

All fractions were desalted into S-300 buffer and assayed for protein and acetyl CoA dependent incorporation of $[^{14}C]HCO_3^-$ into acid-stable products.

Step	Protein	Activity	Specific Activity	Fold Purification	Activity Yield
	mg	*units[b]*	*units/mg*		*%*
Crude extract	215	2.45	0.0114	1	100
30-40% $(NH_4)_2SO_4$	45.1	3.37	0.0748	6.56	138
S-300	10.7	3.35	0.313	27.5	137
Blue Sepharose	1.50	1.86	1.24	109	76
Mono-Q (ACCase I)	0.130	0.720	5.54	486	29

[a]　　Data are from one purification experiment starting with 50 g fresh weight of tissue and are representative of data obtained for eight purifications.

[b]　　Unit = 1 μmol acid-stable product min^{-1}.

B.　　Formation and Specificity of Antibodies to ACCase

Antibodies are sensitive reagents that allow for the identification of gene products from cDNA and other cloned genes. Antibodies to purified ACCase were prepared and used to screen for cDNA clones encoding all or a portion of a gene for ACCase.

Antiserum to maize ACCase was obtained by immunizing a female New Zealand White rabbit (Egli et al., Plant Physiol., 101, 499 (1993)). An intramuscular injection of 100 μg of Mono-Q-purified, SDS-denatured ACCase I in Freund's complete adjuvant was followed by subcutaneous injections of 20 to 100 μg of gel-purified ACCase I polypeptide in acrylamide plus incomplete adjuvant every 4 to 6 weeks, for a total of six injections. Serum was stored at -20°C in 0.02% (w/v) NaN_3.

For Western blots, proteins in SDS gels were electrophoretically transferred to Immobilon (W.B. Parker et al., Plant Physiol., 92, 1220-1225

47

(1990)) for 1 hour at 20 V in a Bio-Rad Transphor semi-dry blotter and then
stained with Ponceau S (E. Harlow et al., "Antibodies - A Laboratory Manual,
Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1988)).
Destained blots were blocked with Tris-buffered saline plus 0.5% (v/v) Tween-
5   20 (Bio-Rad), and 10% (w/v) bovine serum (for antiserum blots only). ACCase
and biotinylated proteins were detected with immune serum (1/10,000) plus goat
anti-rabbit IgG-alkaline phosphatase conjugate or with avidin-alkaline
phosphatase (W.B. Parker et al., Plant Physiol., 92, 1220-1225 (1990)). Blots
were repeated at least three times.

10   For immunoprecipitations, equal ACCase activities (0.58 nmol
min^{-1}) in crude extracts were desalted into S-300 buffer containing 0.1 M KCl
and incubated 1 hour at 25°C with 16 μL buffer or with 16 μL serum consisting
of 0 to 100% ACCase antiserum in preimmune serum. Immune complexes were
incubated 1 hour at 25°C with a 2-fold (IgG binding) excess of Protein
15   A-agarose and then microfuged 5 minutes to obtain immunoprecipitation
supernatant fractions. ACCase activity of supernatants was expressed as a
percent of the 100% preimmune serum control. Data are means plus SE of three
replicate assays for each of two sets of extracts.

                 Western blots and silver-stained gels of purification fractions
20   separated by 7.5% SDS-PAGE showed that neither ACCase I nor ACCase II
Mono-Q fractions contained biotinylated polypeptides smaller than 212 kD. A
polypeptide > 212 kD was the primary protein component of the ACCase I
Mono-Q fragment (Fig. 5). The ACCase II fraction contained a biotinylated
polypeptide > 212 kD and a large amount of a 55 kD non-biotinylated
25   polypeptide. Fractions from earlier purification steps contained additional
biotinylated proteins of approximately 74, 75, and 125 kD (Fig. 5).

                 To better compare the biotinylated polypeptides > 212 kD in
ACCase fractions I and II, we used 6% SDS-PAGE, which showed that the mass
of ACCase II was approximately 8 kD less than that of ACCase I. Molecular
30   masses were estimated to be 219 kD (ACCase II) and 227 kD (ACCase I), based
on comparisons with polypeptide standards and the observation (N.R. Palosaari,
Plant Physiol., 99(S), 359 (1992)) that, on Phastgels (Pharmacia), ACCase I
polypeptide was slightly smaller than dodecameric horse spleen ferritin (238 kD;

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M. Heusterspreute et al., FEBS Lett., 129, 322-327 (1981)). All purification

fractions through the Blue Sepharose step contained both ACCase I and II

polypeptides. Rapid extraction of leaves in buffer containing five additional

protease inhibitors, or a 4 hour incubation of extracts at 25°C, had little or no

5   effect on the relative amounts of the two polypeptides, suggesting that ACCase

II is not a breakdown product of ACCase I.

Antiserum to ACCase I strongly recognized the ACCase I

polypeptide in crude extracts and showed little or no recognition of ACCase II

polypeptides. No bands were recognized by preimmune serum. Assuming that

10   avidin binds similarly to ACCase I and II polypeptides, it appears that the

amount of ACCase II on the Western blot was slightly less than the amount of

ACCase I. However, the relative staining with antibody compared to avidin

indicated that the antibody had significantly less affinity for ACCase II than

ACCase I.

15   To determine whether the same ACCase polypeptides were

expressed in different maize cell types, proteins in mesophyll chloroplasts and

crude extracts of leaves, endosperm tissue, embryos, and BMS cells were

separated by SDS-PAGE. All preparations contained a predominant biotinylated

polypeptide of approximately 227 kD (ACCase I) that was strongly recognized

20   by ACCase antiserum or avidin. Similar 227 kD band densities were observed

when gel lanes were probed with either avidin or ACCase antiserum. The

219 kD ACCase II polypeptide was readily detected in leaves only by avidin

binding, but was in low abundance or not detected in extracts from other tissues.

Only the 227 kD ACCase I polypeptide was detected in purified mesophyll

25   chloroplasts, however, suggesting that the 219 kD ACCase II polypeptide is

localized elsewhere in mesophyll cells or in other cell types of young leaves.

ACCase activity and a > 212 kD biotinylated polypeptide(s) were also found in

bundle sheath strand extracts, but low yields prevented us from determining the

type of ACCase present. Two other major biotinylated polypeptides of 75 and

30   74 kD were found in all tissues. Other non-biotinylated proteins of 66 kD (faint)

and 55 kD were also recognized by ACCase antiserum. The 55 kD polypeptide

was only found in leaves; it was also present in both ACCase I and II Mono Q

fractions (Fig. 5) and was identified as the Rubisco large subunit based on its comigration with protein immunoprecipitated by spinach Rubisco antiserum.

ACCase antiserum immunoprecipitated at least 75% of ACCase activity from crude, desalted extracts of leaves, endosperm tissue, embryos, and
5   BMS cells (Fig. 6), indicating that most of the ACCase activity in these tissues is immunologically related to the ACCase I polypeptide of leaves. Less activity was precipitated from leaves (75%) than from other tissues, particularly embryos (98%). Compared to immunoprecipitation, inhibition of ACCase activity by antiserum in solution was less than 20% as effective in reducing ACCase
10   activity.

The substrate specificity of ACCase from different purification fractions was examined to compare [^{14}C]HCO$_3^-$ incorporation in the presence of different acyl-CoA substrates. Both ACCase I and II utilized propionyl-Co-A 40 to 50% as rapidly as acetyl-CoA at 50 to 500 µM substrate even though they
15   contained no biotinylated polypeptides (Figure 5) the size of known propionyl CoA carboxylases (70 to 75 kD; see E.S. Wurtele et al., Archives of Biochemistry and Biophysics, 278, 179-186 (1990)). Activities in the presence of both acetyl-CoA and propionyl-CoA (250 or 500 µM each) were approximately 90 (ACCase I) to 130% (ACCase II) that of 500 µM acetyl-CoA
20   alone. Crude leaf extracts utilized propionyl-CoA and methylcrotonyl-CoA 60% as efficiently as acetyl-CoA. Methylcrotonyl CoA carboxylase activity was reduced 85% by gel filtration and was completely removed by Blue Sepharose affinity chromatography.

ACCase I and II differed significantly in their inhibition by either
25   haloxyfop or sethoxydim (Fig. 7). Acetyl-CoA or propionyl-CoA-dependent H^{14}CO$_3^-$ incorporation by ACCase I was strongly inhibited (65 to 80%) by 1 µM haloxyfop or 10 µM sethoxydim, while ACCase II activity was inhibited less than 50% for all herbicide/substrate combinations examined.

C.   Cloning and Identification of Maize cDNA Clones Encoding ACCase
30   Maize cDNA clones encoding a portion of the ACCase game were identified by screening a DNA library generated from maize. The cDNA clones were used to identify the sequence of the ACCase gene and to identify the genomic DNA fragments encoding the gene or genes for ACCase.

50

A λ gt11 cDNA library from maize inbred A188 seedlings was prepared by standard method for oligo-dT priming, as described for pea cDNA. (Gantt and Key, Eur. J. Biochem., 166:119-125 (1987). Plaque lifts of the maize cDNA library were screened with maize ACCase antiserum (Egli et al., Plant

5 Physiol., 101, 499 (1993)) to identify plaques expressing ACCase-like proteins, as described by Sambrook et al., cited supra. (1989). The initial screen of 800,000 plaques yielded 120 positives. Rescreening and plaque purification reduced the number of positives to 14. All 14 clones bound ACCase antibodies that, when eluted from plaque lifts (J. Hammarback et al., J. Biol. Chem.,

10 265:12763 (1990)), recognized a 227-kD biotinylated polypeptide on SDS-PAGE western blots of embryo and leaf crude extracts. The strongest western blot reaction was obtained with cDNA clone #15-14. The six best clones were digested with EcoRI to excise maize cDNA inserts. Total insert sizes ranged from 1.2 to 5.1 kb indicating the clones most likely did not contain the full

15 coding sequences for the mature 219-kD and 227-kD ACCase polypeptides (minimum estimates of 6.1 and 6.3 kb, respectively).

Clone #15-14 contained three EcoRI fragments of 2.0, 1.2 and 0.23 kb shown in Figure 8. Southern blots showed that the 1.2 and 2.0-kb fragments of clone #15-14 each hybridized to different fragments in the other

20 five clones, with the exception of clone #4-4 which only contained a 1.2-kb fragment. The six maize cDNA clones contained EcoRI fragments that hybridized to a large transcript (ca. 7.8 kb) on Northern blots of total RNA from maize leaves, embryos and endosperm (Fig.9). BMS cell culture RNA also contained a 7.8 kb transcript, but the hybridization signal is not evident on this

25 exposure (Fig. 9). The relative abundance of the 7.8-kb transcript in embryos was higher than the other sources which is consistent with their ACCase activity.

The three EcoRI fragments were subcloned from cDNA clone #15-14 into BlueScript vector and sequenced by the dideoxy chain termination method (Sequenase 2.0 USB) initially using T3 and T7 primers and then

30 oligonucleotide primers based on insert sequence. A clone #16-6 was also sequenced in a similar manner. Clone #16-6 included three EcoRI fragments of 3.1 kb, 1.2 kb, and 0.23 kb and had additional sequence located upstream from that of clone #15-14. After comparing the sequence and determining that the

51

sequence was the same, the additional 1.2 kb sequence at the 5' end was
sequenced.

Clone #18-5 was sequenced in a similar manner. Clone #18-5
included 3.9 kb, 1.2 kb, and 0.23 kb EcoRI fragments and contains an additional
5   1.9 kb 5' sequence upstream from clone #15-14. Subclone #18-5I (3.9 kb EcoRI
fragment) has been deposited with the American Type Culture Collection and
given Accession No. 69236.

GenBank, PIR-29, and Swiss-Prot 19 data banks have been
searched for amino acid homology with the corresponding amino acid sequences
10   of the three subclones of clone #18-5. Peptide sequences corresponding to the
maize cDNA subclones had higher similarity to chicken, rat, and yeast ACCases
than to any other peptide sequence in the data banks. Figure 8 illustrates the
relative organization of the 3.9, 1.2 and 0.23-kb EcoRI fragments of clone #18-5
and their co-linearity and extent of amino acid identity with chicken ACCase
15   cDNA sequence. This comparison shows that the maize clone #18-5 has a large
region near the 3' end with high amino acid identity (40 to 61%) to chicken
ACCase, a longer region with 23% identity in the middle of the 3.9-kb sequence,
and a short region with 52% identify near the 5' of the 3.9 kb sequence.

Portions of the sequence of the #18-5I subclone have been
20   identified as encoding domains of the ACCase enzyme of functional
significance. Those functional regions include a fragment that spans the
presumed transcarboxylase active site in the enzyme having the following
presumed sequence SEQ ID NO: 2:

25   1112-856
          GTT CCT GCA AAC ATT GGT GGA CCT CTT CCT ATT ACC AAA CCT CTG GAC
          CCT CCA GAC AGA CCT GTT GCT TAC ATC CCT GAG AAC ACA TGC GAT CCA
          CGT GCA GCT ATC TGT GGT GTA GAT GAC AGC CAA GGG AAA TGG TTG GGT
          GGT ATG TTT GAC AAA GAC AGC TTT GTG GAG ACA TTT GAA GGA TGG GCA
30   AAA ACA GTG GTT ACT GGC AGA GCA AAG CTT GGA GGA ATT CCT GTG GGC
          GTC ATA GCT GTG GAG ACA

52

This functional domain is contained in the sequence 1112 to 856 base pair from
the 3' stop codon or carboxy terminus region of the ACCase coding sequence of
maize. This transcarboxylase active sequence is also present in clone #15-14.

Another functional region that has been identified spans the

5   12 base pair sequence encoding the biotin binding site having the following
peptide sequence SEQ ID NO: 3:

5'   GTT ATG AAG ATG   3'
        Val Met Lys Met

10
The biotin binding site is encoded approximately 30% in from the 5' (N-
terminus) end of rat, chicken and yeast ACCase genes. These functional
domains are useful in mapping and further identifying other cDNA and/or
genomic fragments encoding ACCase genes.

15   The cDNA clones encoding portions of the acetyl CoA
carboxylase genes are useful to identify the sequence of the gene or genes and
are useful as probes to locate the genomic copies of the gene or genes. Because
the ACCase antibodies used to screen the λ gt11 library recognize both the 219
and 227 kD ACCase polypeptides, it has not been determined which polypeptide

20   is encoded by these less than full length clones. It is likely that the majority of
the clones encode the 227 kD polypeptide since that polypeptide is more
abundant in the leaf tissue source of the DNA library and the antibodies have a
higher affinity for the 227 kD ACCase polypeptide.

EXAMPLE VI
25   **Isolation and Sequencing of Genomic Encoded ACCase Genes
and a Complete cDNA Sequence of a Maize ACCase Gene**

The maize genome has been analyzed to identify copy number
and location of the genomic copies of ACCase gene or genes. Four distinct
types of maize ACCase genomic clones have been identified, termed A1, A2, B1

30   and B2 (see below).

To obtain genomic copies of ACCase genes, a maize B73
genomic library (Clontech, Palo Alto, CA) was screened with the 2 kb subclone
from #15-14 and several clones of about 15 kb were identified as having
homology to the ACCase cDNA. Restriction mapping and partial sequence

35   analysis revealed two types of genomic clones (Type A and Type B) that differed

in restriction sites and in their position relative to the ACCase partial cDNA
sequence as shown in Figure 8.

The 2.5 kb EcoRI-SalI fragment (#16) from the Type A genomic
clone and the 3.0 kb EcoRI-EcoRI fragment (#34) from the Type B genomic

5   clone were shown to hybridize to the 3.9 kb probe from #18-5 and were
subcloned into the Bluescript vector and sequenced. Approximately 1.5 kb of
DNA sequence from the genomic Type A 2.5 kb fragment were 100% identical
to coding sequence from the 3.9 kb cDNA subclone #18-5I described in Example
V; the remaining sequence exhibited no identity with the cDNA clone and

10   presumably represents a noncoding intron sequence. A 350 nucleotide sequence
derived from the genomic Type B 3.0 kb fragment was about 95% identical to
the cDNA clone indicating that its coding sequence differs from that of genomic
Type A. These results also indicate that the maize genome encodes at least two
different genes encoding a polypeptide having acetyl CoA carboxylase activity.

15   To identify and clone the remainder of the gene representing the
amino-terminus of maize ACCase, additional regions from the Type A genomic
clone have been subcloned and partly sequenced. The 3.5 kb HindIII-HindIII
fragment (#28) has been sequenced for about 400 nucleotides from each end.
The 3' end of #28 shows significant homology to the amino acid sequence from

20   the chicken sequence located about 0.5 kb from the start of the chicken gene.

The complete sequence for fragment #28 can be obtained and
analyzed to determine whether it contains the 5' end of the ACCase coding
region. The start of the transcribed region, and thus the likely start of the coding
region for ACCase, can be identified by using the genomic clones in RNAse

25   protection analysis (J. Sambrook et al., "Molecular Cloning - A Laboratory
Manual," Cold Spring Harbor Press, Cold Spring Harbor, New York (1989)).
Based on sequence data from the genomic clone, alignment, as shown in Figure
8, with sequences of other ACCases and identification of potential open reading
frames, oligonucleotide primers can be constructed to synthesize cDNA

30   molecules representing the amino terminus of the ACCase gene. These
molecules can be hybridized to genomic Type A DNA fragments such as #28
and the nonhybridizing portions digested with S1 nuclease. The end of the
protected fragment are determined by analysis on a DNA sequencing gel.

54

To synthesize the remaining coding region between the end of the cDNA clone #18-5 and the start of transcription, two oligonucleotide primers were synthesized. Primer 1 is complementary to the DNA sequence: (SEQ ID NO:4)

5   5' GCCAGATTCC ACCAAAGCAT ATATCC 3'

near the 5' end of cDNA subclone #18-5I and can be used as a primer for synthesis of cDNA molecules from maize seedling, leaf or embryo RNA.

A primer corresponding to a DNA sequence near the transcription

10   start site can be used in combination with primer 1 for the amplification of DNA by the polymerase chain reaction (PCR). Several independent clones are then sequenced and their sequences compared to the known sequence of the Type A genomic clone to determine the exact coding sequence corresponding to that maize gene for ACCase. A similar strategy can be used to obtain the complete

15   coding sequence for genomic Type B ACCase.

The remaining cDNA sequence was obtained by three successive rounds of RT-PCR using oligonucleotide primers based on genomic apparent exon (5') and known cDNA (3') sequences. The primers used to amplify nucleotides 1-240 of the cDNA were 28sst-a5+ (SEQ ID NO. 7) and

20   28sst-6at3+ (SEQ ID NO:8), nucleotides 217-610 of the cDNA were 28sst-5+ (SEQ ID NO:9) and 28-2t3+ (SEQ ID NO:10), and nucleotides 537-2094 of the cDNA were ACCPCR5' (SEQ ID NO:11) and I55⁻ (SEQ ID NO:30) (Table V). PCR products corresponding to nucleotides 1-240, 217-610, and 537-2094 of the final sequence were cloned into PCR-script (Stratagene).

TABLE V

cDNA Position	5' primer designation	5' primer sequence	3' primer designation	3' primer sequence
nt-1-240	28sst-a5+	GGTCTTCAATTGTGCTGTCTGG (SEQ ID NO:7)	28sst-6at3+	CCTTGACGAACAGACTGGCTGTGC (SEQ ID NO:8)
nt 217-610	28sst-5+	CACAGCCAGTCTGTTCGTCAAGG (SEQ ID NO:9)	28-2t3+	CCTCTACGTAATTGGTCAGC (SEQ ID NO:10)
nt 537-2094	ACCPCR5'	CATAGCTATGGCAACTCCGG (SEQ ID NO:11)	155-	GGATATATGCTTTGGTGGAATCTGGC (SEQ ID NO:30)

56

The original 5.4-kb cDNA clone #18-5 and PCR products from at
least three individual PCR per oligonucleotide pair were sequenced in both
directions by the dideoxy chain-termination method, using either Sequenase II
(U.S. Biochemicals) or ABI 373 (Applied Biosystems, Inc.) protocols. No

5   sequence differences were found in regions of clone overlaps. The complete
sequence of the cDNA of maize ACCase (nucleotides 1-7470 SEQ ID NO:5) and
its corresponding amino acid sequence (amino acids 1-2325 SEQ ID NO:6) are
shown in Figures 13 and 14. The 7470 bp cDNA includes a 459 nucleotide 3'
untranslated region and 36 nucleotides of 5' untranslated sequences.

10   The first Met codon in the cDNA (nucleotides 37-39) was
identified as the start codon based on its similarity to consensus initiation
sequences (Kozak, J. Cell. Biol., 108, 229 (1989); Lutcke et al., Embo. J., 6, 43
(1987)). An in-frame stop was found in the genomic sequence 6 nucleotides
upstream of the sequenced cDNA, and RT-PCR analysis of this region suggested

15   that the in frame stop codon was also present in the cDNA. The 3' end of the
coding sequence was defined by two stop codons present in the large open
reading frame after nucleotide 7011. The translated coding sequence predicted a
polypeptide of 2325 amino acids (257 kD; SEQ ID NO:6) which was 79 to 81%
identical to the multifunctional (MF) ACCases from alfalfa (Shorrosh et al.,

20   Proc. Nat'l. Acad. Sci., 91, 4323 (1994)) and wheat (Gornicki et al., Proc. Nat'l
Acad. Sci., 91, 6860 (1994)), and to a 118-amino acid predicted polypeptide of a
rice expressed sequence tag (Genbank accession # D39099, T. Sasaki), but only
53 to 55% identical to ACCase from other eukaryotes.

In a pileup alignment of plant ACCases (Genetics Computer

25   Group, Madison, Wisconsin), Met 1 of both maize and *Brassica napus* ACCases
was located about 130 amino acids upstream of the conserved sequence
VDEFCKALGG, compared to only 25 amino acids upstream for other plant
ACCases. The predicted 2325 amino acids of maize ACCase contains a
biotinylation site at position 806, within the conserved MKM motif (Ton et al.,

30   Eur. J. Biochem., 215, 687 (1993)). The arrangement and amino acid sequence
of binding sites (Shorrosh et al., Proc. Nat'l. Acad. Sci., 91, 4323 (1994)) for
ATP (amino acids 318-333), biotin (amino acids 799-811; biotin at 806),

acetyl-CoA (amino acids 1952-1961), and carboxybiotin (amino acids
1662-1711) were highly conserved among all MF ACCases.

EXAMPLE VII

Characterization of other Genomic Clones

5   The initial restriction fragment length polymorphism (RFLP)
analysis of EcoRI-digested total DNA from three maize inbred lines showed one
band when probed with the 2 kb subclone from #15-14 (internal to gene) and two
bands when probed with the 1.2 kb subclone (near the 3' end of the gene).
Fragments homologous to the 2 kb probe were monomorphic and the more

10   intense of the two bands hybridizing with the 1.2 kb probe was dimorphic. As
discussed in Example V, these results support the view that maize contains at
least two distinguishable ACCase genes and that they may be quite similar for
much of the coding region. Additional genomic Southern blots of a set of
recombinant inbred lines were used to map polymorphisms for the ACCase

15   probes to maize chromosomes. One polymorphism was mapped to the short arm
of chromosome 2; other polymorphisms were not evident in these initial tests to
identify a chromosomal location for other maize ACCase genes.

        The isolation and restriction mapping of additional genomic
clones from a B73 genomic library (Clontech) resulted in the identification of

20   four different types of clones termed A1, A2, B1 and B2 (Figures 16-19) which
had 96% nucleotide sequence identity. Types A and B correspond to previously
published pA3 and pA4 cDNAs (Ashton et al., Plant Mol. Biol., 24, 35 (1994))
and differ from pA3 and pA4 by ~ 4% in their coding sequences.

        Type A and B genomic clones have linear sequence homology

25   except for an insertion in an intron of the Type B genes about 1400 bp 3' of the
A1(SEQ ID NO:5) translation start site. Analysis of the insert boundaries
revealed a 3-bp target site duplication and a 6-bp direct repeat, and further
sequence analysis showed the presence of two new and unique LINE elements
(Long Interspersed Nuclear Elements) in B1 and B2. Mammalian LINE

30   elements are highly abundant (10^4 to 10^5 copies), 6 to 7 kb long, and have
frequent 5'-end deletions and an A-rich 3' terminus. They are flanked by short
direct repeats, and contain two ORFs, one encoding a reverse transcriptase.
Three LINE elements (Cin4, 50-100 copies in maize; del2, 250,000 copies in

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lily; BNR1, 2-5% of genome in sugarbeet) have been described in plants (Leeton et al., Mol. Gen. Geneti., 237, 97 (1993); Schmidt et al., Chromo. Res., 3, 335 (1995); Schwarz-Sommer et al., EMBO J., 6, 3873 (1987)). Maize ACCase B1 has one unique LINE element and B2 has two. The two B2 LINE elements were

5   characterized by differences in their reverse transcriptase sequence. The B genomic clone inserts have characteristic LINE features including cysteine motifs and a possible polyA tail, and high abundance. The LINE insert also has been found in an intron of the maize *Shrunken-2*-gene (Hannah et al., Plant Physiol., 98, 1214 (1992)).

10   The partial nucleotide sequence (3489 nucleotide) of a Type A1 ACCase genomic clone is shown in Figure 16 (SEQ ID NO:12). The clone is a HindIII fragment which includes nucleotides 1-931 of the cDNA in Figure 13 (SEQ ID NO:5), and the first four introns within the coding region, at positions 240 (460 nucleotides), 296 (480 nucleotides), and 872 (76 nucleotides) of SEQ

15   ID NO:5. The clone also has 1395 nucleotides 5' to the cDNA of SEQ ID NO:5 (i.e., 1431 nucleotides 5' of the translational start at nucleotide position 1432).

           The partial nucleotide sequence (1328 nucleotide) of another Type A clone is shown in Figure 17 (SEQ ID NO:13). The partial sequence is all 5' untranslated sequence and contains a 7 base insert between nucleotides

20   279-290, but is otherwise identical to SEQ ID NO:12.

           The partial nucleotide sequence of six Type A2 clones is shown in Figure 18 (1565, 1168, 638, 558, 976 and 852 nucleotides; SEQ ID NOs 14, 15, 16, 17, 18 and 19, respectively).

           Within the A1-A2 clone pair, identified differences are in introns

25   and 5' UTR sequences. The A2 genomic clone is weakly amplified with Type A1 PCR primers specific for the 5' UTR if the 3' primer employed is for a conserved amino acid sequence found in all ACCases (e.g., 28sst-110F, ACTGTGCGTTTGAGAAGGTC, SEQ ID NO:28, and 28sst-2T3+, CCTCTACGTAATTGGTCAGC, SEQ ID NO:29). The A2 amplified product is

30   the same size as that from the Type A1 genomic clone, and restriction analysis indicated a difference in sequence from A1. Sequence differences in the 5' region should provide a means to distinguish between expression of A1 and A2 ACCase genes and to determine whether A2 also encodes a CTP.

The partial nucleotide sequence (231, 207 and 180 nucleotides) of three Type B clones is shown in Figure 19 (SEQ ID NOs 20, 21 and 22, respectively).

The cDNAs corresponding to genomic clones A2, B1 and B2 are

5   cloned and sequenced in a manner similar to that described above. The derived amino acid sequences are aligned with known ACCase sequences. If putative CTP sequences are identified, functionality is tested as described below. Also if the tissue specificity and developmental timing of expression differ for different ACCase genes, the sequences of the promoter regions of the corresponding

10   genomic clones are compared. Gene-specific probes for specific ACCase genes can provide more information on their roles in lipid synthesis (plastid and cytoplasmic isoforms), secondary metabolism (cytoplasmic isoforms), and herbicide resistance (likely plastid isoforms).

A 3' Type A1 ACCase cDNA probe mapped to chromosome 2S

15   (Egli et al., Maize Genetics Newsletter, 68, 92 (1994)) and to 10L (Caffrey et al., Maize Gen. Coop., 69, 3 (1995)). Two 5' Type A1 cDNA probes which span the transit peptide mapped to chromosome 2S in the same location as the 3' probe (see maize genetic map, 1996 version, Maize Genomic Database). PCR primers 28sst-97F (CCTTTTTATGGCACTGTGCG, SEQ ID NO:25) and 28sst-6t3+;

20   (CATCGTAGCCTATATGAGGACG, SEQ ID NO:26) located in non-coding regions of A1 that span the chloroplast transit peptide were used to amplify a B73 chromosome-specific product which segregated with the resistance trait. A nearby 5' primer (28sst-a5+, SEQ ID NO:7) amplified all genotypes and functioned as a positive control. Herbicide resistance due to the *Acc1-S3*

25   mutation segregates (29/29 individuals to date) with production of a Type A 5' end-specific PCR product derived from the mutant parent while herbicide sensitive plants lack the transit sequence (15/17 progeny). Two individual plants which contained B73-specific DNA at this location died of unknown causes while grown in the presence of herbicide.

30   Mutations in maize that confer resistance to cyclohexanedione and aryloxyphenoxypropionate herbicides by means of an altered ACCase target are found at two non-allelic loci, *Acc1* and *Acc2*. A1 and A2 appear to encode plastidic ACCases and correspond to the *Acc1* and *Acc2* herbicide resistance

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loci. *Acc2* has been mapped to 10 L (VanDee, M.S. Thesis, University of
Minnesota (1994)). *Acc1* is the site of five allelic mutations including *Acc1-S2*
and *-S3* (Marshall et al., Theor. Appl. Genet., 83, 435 (1992)), and has been
mapped to chromosome 2.

5   Only one plastidic ACCase polypeptide was identified by SDS-
PAGE of maize leaf extracts, although 2-D gel analyses might provide evidence
for a second, highly similar isoform. Of the two ACCase isoforms, only
ACCase I shows altered herbicide inhibition in *Acc1-S2* mutants, and most of the
ACCase activity in leaves and developing embryos is herbicide-resistant and
10   thus attributed to the *Acc1-S2* gene product.

Although a 3' ACCase probe has been mapped both to 10L near
Acc2-S5 and to 2S, the conserved sequence of ACCase genes and lack of
polymorphism in multiple bands complicates identification of genes encoded at
these loci. The Type A1 ACCase gene is probably located on chromosome 2,
15   since (i) 5' untranslated and chloroplast transit peptide probes from Type A1
hybridize to two bands (dark and light) in maize inbreds, and (ii) analysis of
maize-oat addition lines carrying maize chromosomes 2 through 9 indicates the
dark band is on chromosome 2 and the light band is on chromosome 1 or 10.

Type B ACCase genes are likely to encode cytosolic isoforms.
20   Given that cytosolic malonyl-CoA is a precursor in the synthesis of many
secondary metabolites including flavonoids (e.g. maysin, a corn silk component
associated with corn earworm resistance), these cytosolic ACCases can have
agronomic utility.

Northern blot analysis of total maize RNA with an ACCase probe
25   (nucleotides 3400-5932) showed a single 8.3 kilobase band. To determine
whether the expression of ACCase RNAs was developmentally regulated, blots
of total RNA from 16 to 42 DAP (days after pollination) embryos were probed
with an ACCase cDNA fragment. Transcript abundance peaked about 23 DAP
and the steady state pattern was similar to *in vitro* ACCase enzyme activities and
30   protein measured from developing embryos. Type A- and B- specific ^{32}P-CTP-
labeled antisense transcripts were 780 nt long (662 nt of ACCase sequence + 118
nt of vector/promoter sequence) and were identical except for 15 base
mismatches scattered along their length. Each antisense transcript was

hybridized to total RNA from embryos at 16, 20, 23, and 42 DAP and digested
with RNAse A/TI mixture to yield a 662-base fragment specific to the probe
used. The results showed that the Type A transcript was more abundant than
Type B at all tested stages, and that only Type A remained high in older
embryos. Types A and B had similar expression patterns and peaked
around 20-23 DAP. The ratio of Type A:B mRNA in leaves was about 2:1,
similar to its relative abundance in cDNA expression libraries.

EXAMPLE VIII

Expression of the Maize ACCase Chloroplast Transit Peptide

The N-terminus of the predicted maize ACCase polypeptide is
longer than that of predicted cytosolic ACCase isoforms and has several
characteristics typical of chloroplast transit peptides within the first
approximately 73 amino acids of the predicted N-terminal sequence. The CTP
cleavage site motif is not found in the putative maize ACCase CTP, although
only about 30% of known CTPs contain this consensus sequence (Gavel and von
Hejne, FEBS Lett., 261, 455 (1990)). However, the maize ACCase N-terminus
appears to have several other properties typical of known CTPs: (1) a lack of
acidic residues in amino acids 1-49, (2) high Ser + Thr content (69% within
amino acid residues 23-35), (3) an R-rich region between S- and D-rich regions
in amino acid residues 36-49, and (4) a predicted turn →β sheet within amino
acid residues 58-73 (von Hejne and Nishikawa, FEBS Lett., 278, 1 (1991)).

The ability of the amino acid sequence contained within the
N-terminal 100 amino acids of the translated maize acetyl-CoA carboxylase
(ACCase) cDNA to direct the N-terminal portion of the maize ACCase biotin
carboxylase domain into chloroplasts was tested *in vitro* by methods used
extensively in the literature (see Cline et al., J. Biol. Chem., 260, 3691 (1985);
Lubben and Keegstra, Proc. Nat'l. Acad. Sci., 83, 5502 (1986)). The criteria for
import was that (1) *in vitro*-synthesized, ^{35}S-labeled protein was imported into
chloroplasts, and (2) the transported protein was smaller than the original
translation product, by an amount which corresponds to the removal of the
expected CTP. Import studies utilized either maize or pea chloroplasts. Pea
chloroplasts have been reported to correctly import proteins from many different
species, including maize (Nieto-Sotelo et al., Plant Physiol., 93, 1321 (1990)).

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Alternatively, the function of the putative maize ACCase CTP is tested by inserting the first 258 coding nucleotides of maize ACCase in frame with and 5' of a GUS reporter gene in pBI221 (Clontech). This construct and the pBAR plasmid are used to co-transform maize "Black Mexican Sweet" suspension cells

5   by particle bombardment. Basta-resistant transformants are selected, and GUS activity and/or protein is assayed in surviving cultures or in plasmids isolated from transformants.

A partial ACCase construct consisting of nucleotides 1-833 of SEQ ID NO: 5 including the putative CTP (nucleotides 37 to 256) and the first

10   domain within the biotin carboxylase region (identified by amino acid sequence comparison with E. coli biotin carboxylase; see Waldrop et al., Biochem., 33, 6249 (1994)) was amplified by PCR and cloned into the EcoRV site of PCR-script (Stratagene) to create the plasmid pBCN1. A corresponding plasmid lacking CTP sequences (nucleotides 278-833) was also made (-pBCN1). The

15   protein encoded by -pBCN1 begins at amino acid residue 83 (Val → Met).

Constructs were transformed into E. coli SURE cells (Stratagene). Restriction analysis of pBCN1 with BamH1 and HindIII indicated that the 5' end of the ACCase was located adjacent to the T7 RNA polymerase binding site in PCR-Script. A partial sequence of pBCN1 obtained by using the T7 sequencing

20   primer and the ABI373 automated sequencing protocol confirmed this orientation and showed that the pBCN1 insert sequence was identical to maize ACCase cDNA for at least the first 300 nucleotides and that it included the maize ACCase Met 1 ATG. An acyl carrier protein clone containing a CTP (spinach ACPII, a gift of Dr. John Ohlrogge, Michigan State University) can be

25   used as a positive control. These constructs are used for *in vitro* transcription, translation, chloroplast import, and SDS-PAGE analysis of products in the same manner as pBCN1.

Purified pBCN1 was digested with EcoRI to linearize the plasmid at the 3' end of the BCN1 insert, electrophoresed in 1.5% agarose, and the

30   plasmid band at approximately 3.8 kb was excised and Gene-Cleaned (BioLab 101). The purified band was digested with 20 μg proteinase K to remove any residual RNAse, extracted with phenol and then chloroform under RNAse-free conditions. DNA content was estimated by ethidium bromide fluorescence in

droplets, relative to λ DNA standards (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed. (1989). One μg of pBCN1 DNA was transcribed into capped RNA with the T7/mMessage mMachine kit (Ambion). Uncapped transcripts (Sp6 RNA polymerase; Promega) of pea RUBISCO small subunit

5   (SSU; Anderson et al., Biochem. J., 240, 709 (1986)) were also transcribed. RNA yield was estimated by determining the % incorporation of a ^{32}P-ATP (Amersham) into a precipitable product, according to the Ambion kit instructions. Electrophoresis and autoradiography of ^{32}P-labeled product showed that it contained a single RNA band of approximately 895 nucleotides, as

10   expected.

The RNA transcripts were translated into ^{35}S-labeled polypeptides with Ambion's wheat germ IVT kit and approximately 45 μCi ^{35}S-methionine (Amersham; 37 TBq/mmol) in a 50-μl reaction. Labeled proteins were held on ice 6 hours prior to their use in chloroplast import experiments.

15   Pea (cv. "Little Marvel") and maize (inbred A188) plants were grown in a growth chamber at 25°C, 16 hour day length. Chloroplasts were isolated from pea and maize leaves 7 days after planting, respectively, as previously described (Burton et al., Pestic. Biochem. and Physiol., 34, 76 (1989); Egli et al., Plant Physiol., 101, 499 (1993)). Intact mesophyll chloroplasts were

20   washed in resuspension buffer [50 mM HEPES-KOH, pH 7.8 plus 0.33 M (pea) or 0.66M (maize) sorbitol] in preparation for import assays. Suspensions were diluted to obtain 75 μg chlorophyll/0.3 ml (Arnon, Plant Physiol., 24, 1 (1949)).

Import experiments were carried out essentially as described by Cline et al.(J. Biol. Chem., 260, 3691 (1985)). Import reactions containing 0.3

25   ml pea or maize chloroplast suspension, 40 μl ^{35}S-translation mixture, 3 mM Mg-ATP and 10 mM Met were incubated under light for 1-30 minutes at 25°C. Unimported proteins were digested for 30 minutes with 40 μg of thermolysin, and proteolysis was stopped with 10 mM EDTA.

Chloroplasts were re-isolated by centrifuging them through 1-ml

30   40% v/v Percoll gradients in the presence of resuspension buffer plus 3 mM Mg-ATP, 10 mM Met, and 20 mM EDTA, washed twice in the same buffer, and resuspended in 65 μl of 1 mM MgCl$_2$/10 mM Tris buffer, pH 8.0. Chloroplasts were lysed by three cycles of freeze-thawing in liquid N$_2$, microfuged, and

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aliquots of the supernatants and of the original *in vitro*-translated proteins were analyzed by SDS-PAGE in 8-25% gradient Phast gels (Pharmacia), followed by direct detection of radiolabeled proteins in the wet gels (AMBIS) (Figure 20).

As estimated by SDS-PAGE, a 30 minute import converted the
5   original 32-kD BCN1 polypeptide to a doublet of 27.2 and 27.5 kD in maize and produced an additional 30-kD band in pea (Figure 20A). Neither maize or pea chloroplasts imported -pBCN1-derived polypeptides. Formation of the 27.2-kD polypeptide likely resulted from cleavage after amino acid #47-49, a likely cleavage site because it lies between S- and D-rich regions, and R residues are
10   located at -2, -7, and -8 (S. Gavel and G. Von Heijne, FEBS Lett., 261, 455 (1990)).

Time-dependence of BCN1 import was further examined (1-30 minutes) (Figure 20B) to determine if any imported polypeptides were a result of incomplete processing or proteolysis. Import was maximal after 15
15   minutes, but import time had no effect on the relative amounts of different-sized import products. Higher amounts of ATP (5 mM) stimulated import relative to lower amounts of ATP (< 0.2 mM). The data suggest that, in maize, efficient cleavage of BCN1 occurs at two closely adjacent sites and that partially processed products are also formed during BCN1 import by pea chloroplasts.
20   Therefore, nucleotides 1-833 of the maize ACCase gene encode a CTP.

EXAMPLE IX
Expression of a cDNA Clone or Genomic Clones Encoding the ACCase Gene

The cDNA and genomic clones encoding all or a portion of the
25   ACCase gene can be subcloned into a known expression system and the gene products reactive with the antibodies specific for maize ACCase can be identified using a Western blot. For example, the ACCase cDNA clones are inserted into two transformation plasmids: (i) Glb1exp which contains the embryo-specific maize Globulin1 (*Glb1*) promoter and 3' regions (Belanger et
30   al., Genetics, 129, 863 (1991)); and (ii) pAHC17 which contains the maize ubiquitin (*Ubi-1*) constitutive promoter and first exon and intron, and the NOS 3' terminator (Christensen et al., Plant Mol. Biol., 18, 675 (1992); Toki et al., Plant Physiol, 100, 1503 (1992)). The 3' end of the A_1 cDNA has a unique *Sal*I site

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just 3' of the stop codon which is used to ligate into a *Sal*I site in both plasmids ahead of the construct terminator. Other cloning sites will be added as needed to the plasmids or cDNA to complete the ligation of the 5' end. The gene products can also be further characterized structurally and/or enzymatically. This will

5   ensure that the genomic and cDNA clones that encode acetyl CoA carboxylase can be screened for promoters that provide for overproduction of the native or herbicide tolerant ACCase enzyme in plants.

For example, the 2 kb EcoRI fragment from clone #15-14 can be subcloned into a plant transformation plasmid pBI121 or pBI221 downstream

10   from the 35S CaMV promoter and upstream from the nopaline 3' polyadenylation signal sequence, as described in Jefferson, Plant Molec. Biol. Reptr., 5, 387-405 (1987). This plasmid can then be used to transform plant cells such as tobacco, Brassica and Arabidopsis cells using protoplast or biolistic transformation, as described by W.J. Gordon-Kamm et al., Plant Cell, 2, 603-618

15   (1990); M.E. Fromm et al., Bio/Technology, 8, 833-839 (1990); An, Methods in Enzymology, 153, 292 (1987); and D'Hafluin, The Plant Cell, 4, 1495 (1992). An increase in transient expression can be detected using quantitative Western blotting with antibodies specific for the ACCase enzymes. Polyclonal antibodies to maize ACCase most likely do not substantially crossreact with ACCase from

20   dicots like tobacco or Arabidopsis.

Alternatively, the ACCase gene can be subcloned along with the 35S Came promoter into a binary Ti vector pGA482, as described in An, cited supra., which is a binary Ti vector system and can be used to transform plant cells by Agrobacterium-mediated transformation. Stably transformed plants can

25   be generated by standard methods as described in Example III, and levels of expression of ACCase genes can be determined by quantitative Western blots, as described in Harlow and Lane, Antibodies, Cold Spring Harbor Laboratories (1988). The ability to monitor expression of cloned ACCase genes will permit the identification of promoters that provide for enhanced expression of the

30   ACCase gene. The expression system can be used to screen for those promoters that enhance gene expression of the ACCase gene at least about 5 to 10-fold over the endogenous levels of ACCase produced normally in the plant cells. Because the 35S Came promoter is known as a strong promoter, it is likely this promoter

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will provide for at least a 5-fold increase in the expression of ACCase over that
normally produced in the plant cell.

In addition, this expression system can be used to screen antisense
DNA sequences. For example, an antisense sequence can be obtained that is
5   complementary to an about 0.5 kb region of the maize ACCase cDNA that has
high homology with a portion of the chicken ACCase gene and contains the
sequence for the presumed transcarboxylase active site domain, as shown in FIG.
8. The antisense sequence could be subcloned into a pBI121 or pBI221
expression under the control of an inducible plant promoter, such as nitrite
10   reductase promoter (Back et al., Plant Molec. Biol., 17:9-18 (1991)). The
ability of the antisense sequence to inhibit expression of the native ACCase gene
can be evaluated in transformed cells, for example as described in Hamilton et
al., Nature, 346:284-287 (1990).

EXAMPLE X
15   Identification and Cloning of the Gene From Herbicide Resistant Maize
Cell Lines

Herbicide resistant maize cell lines were generated as described in
Examples I, II, and IV. These herbicide resistant cell lines have been shown to
produce an ACCase enzyme that is less sensitive to inhibition by sethoxydim or
20   haloxyfop. The genes encoding the herbicide resistant forms of the ACCase will
be identified and cloned using standard methods as described in Sambrook et al.,
Guide to Molecular Cloning: A Laboratory Manual (1989). The genes encoding
the herbicide resistant forms of ACCase can then be introduced into herbicide
sensitive plant species by standard methods to confer herbicide resistance . For
25   example, the ACCase enzyme in the maize cell line 2167-9/2160-154 S-1 is at
least 100-fold less sensitive to sethoxydim than the wild-type.

DNA from the cell line or plants will be obtained and digested
with EcoRI and/or other appropriate restriction enzymes, according to standard
methods. The restriction enzyme digest will be separated out by agarose gel
30   electrophoresis and probed with either the 2 kb or the 3.9 kb cDNA ACCase
probe described in Example V. Fragments hybridizing to the 2 kb or 3.9 kb
probe will be subcloned into a Bluescript vector and portions of the gene will be

sequenced, as described in Example V, to verify that the entire ACCase gene has been isolated.

To confirm that the clone encodes the ACCase gene, it will be subcloned into the pBI121 or pBI221 expression vector, as described in Example

5   VIII. The ACCase gene product expressed by the clone in either Black Mexican sweet corn cells or tobacco cells will be evaluated for reactivity with ACCase specific antibodies, by enzyme activity, and/or resistance of the enzyme activity to inhibition with sethoxydim and/or haloxyfop. It is likely that the cloned gene will encode an ACCase which is resistant to inhibition by sethoxydim and

10   haloxyfop. This gene can then be introduced into an herbicide-sensitive embryogenic plant cell or an embryo, including maize cells or immature embryos, to confer herbicide resistance to that plant species upon regeneration.

The complete coding sequence encoding the herbicide resistant form of the ACCase enzyme will be cloned into a plant transformation vector

15   such as pBI121 or pBI221 as described in Jefferson, Plant Molec. Biol. Reporter, 5:387-405 (1987). This vector contains the 35S Came constitutive promoter, the β-glucuronidase structural gene, and the nopaline synthase 3' polyadenylation signals. The β-glucuronidase gene is replaced with a cloned ACCase gene. Optionally, the cloned ACCase gene can be combined with natural or

20   synthetically produced chloroplast transit peptide sequence from pea, as described in Keegstra & Olsen, Ann. Rev. Plant. Physiol./Mol. Biol., 40:471-501 (1989) and/or unique restriction sites introduced so the cloned gene can be distinguished from the endogenous maize ACCase gene. Standard methods of subcloning will be utilized as described in Sambrook et al., cited supra.

25   For transformation of maize cells, type II calli can be transformed using biolistic transformation, as described by W.J. Gordon-Kamm et al., Plant Cell, 2, 603-618 (1980); M.E. Fromm et al., Bio/Technology, 8, 833-839 (1990); and D.A. Walters et al., Plant Molecular Biology, 18, 189-200 (1992). Alternatively, type I embryogenic calli can be transformed using electroporation

30   after mechanically or enzymatically wounding calli, as described by D'Hafluin et al., The Plant Cell, 4:1495 (1992). Once the cloned gene is introduced into these cells and transformants are selected, typically by antibiotic resistance, fertile transgenic maize plants can be regenerated, as described by D'Hafluin et al. cited

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supra. Fertile transgenic plants can be evaluated for herbicide tolerance, as described in Example III. It is likely that the fertile transgenic plants having and expressing a cloned ACCase gene as an ACCase resistant to sethoxydim and/or haloxyfop will exhibit herbicide tolerance as compared to the corresponding

5   untransformed plant.

EXAMPLE XI
Generation of Transgenic Plants Having an Increase in Oil Content

Once identified and cloned, the gene or genes from maize acetyl CoA carboxylase can be introduced into monocot or dicot plant species,

10   including maize, under the control of a promoter that provides for overexpression of the ACCase enzyme. The overexpression of the ACCase enzyme is likely to lead to an increase in the oil content of the plants and seeds.

Naturally occurring soybeans that have a high oil content and soybeans that have a low oil content have been identified. The acetyl CoA

15   carboxylase from both types of soybeans was isolated, as described in Example V. The activity of the enzyme was measured as a function of the time of seed development and the results are shown in Figure 11.

The results in the Figure 11 indicate that higher oil content soybean is associated with a 2-fold increase in the ACCase activity during early

20   to mid stages of development when compared with a low oil content soybean. Thus, increased expression of the ACCase gene correlates with an increase in the oil content of the seed. Total oil content of the seed was also measured at maturity (60 days). The high oil producing cell lines, Anoka and PI28C.134, have a total oil content of 21.8% and 19.9%, respectively. In contrast, the low

25   oil soybean line of M76-395, has an oil content of 13.6% oil. Thus, the increase of ACCase expression early in seed development correlates with a higher total oil content in the seed at maturity.

A gene encoding a genomic maize acetyl CoA carboxylase can be isolated, as described in Example V, and used to transform plant species by

30   protoplast or biolistic transformation. If the gene is combined with a strong promoter, such as the 35S cauliflower mosaic virus promoter, overexpression of the ACCase gene is likely. Alternatively, selecting transformed cells with multiple copies of the gene can also result in transformed cells overexpressing

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the ACCase gene. The gene can be cloned into a vector such as pBI121 or
pBI221, as described by Jefferson, cited supra. This vector contains the 35S
cauliflower mosaic virus promoter, the β-glucuronidase structural gene, and the
nopaline synthase 3' polyadenylation signals. The cloned ACCase gene can

5   replace the β-glucuronidase gene and then be used to transform plant cells,
including maize, as described in Example VIII.

Transformed cells can be screened for overproduction of ACCase.
The presence of the cloned gene can be verified by identifying the unique
restriction enzyme sites incorporated into the cloned gene. ACCase levels can

10   be assessed by standard enzyme assay methods and quantitative Western blots
using antibodies specific for maize ACCase. Fatty acid and lipid content in cells
lines overproducing ACCase are likely to be elevated and can be assessed using
standard methodologies, as described in Clark & Snyder, JACS, 66:1316 (1989).
Transformed cell lines overproducing ACCase and having increased total oil

15   content will be used to regenerate fertile transgenic plants and seeds, as
described in D'Hafluin, cited supra.

EXAMPLE XII

Generation of Transgenic Plants Having an Increase in Plastidic ACCase Activity

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Maize embryos are transformed with sense and antisense cDNA
constructs encoding the plastidic A1 ACCase. Selected transgenic cultures and
regenerated transgenic plants and progeny are subjected to detailed analyses of:
ACCase transcript levels; activity of ACCase I (plastidic) and ACCase II

25   (presumably cytosolic) in various plant tissues; fatty acid synthesis; lipid
accumulation in kernels (primarily embryos); and other plant traits. The
culmination of these tests indicates whether plastidic ACCase activity can be
modified via transformation and whether fatty acid synthesis is affected.

1.   Transformation of maize embryos and plant regeneration

30   Immature maize embryos of the Hi-II genotype are transformed
by particle bombardment according to slight modifications from previously
described procedures (Fromm et al., Biotech., 8, 833 (1990); Koziel et al.,
Biotech., 11, 194 (1993)). This procedure has been employed in transformation
studies for bombardment of approximately 15,000 embryos which were then

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selected for Basta-resistant callus (*bar* selectable marker gene expression), and regenerated into plants. Transformed (Basta-resistant) plants are obtained from 1-2% of the initial embryos and, when separate plasmids are used for co-transformation, the nonselected transgene is recovered in about 50% of the

5   Basta-resistant plants.

Basta-resistant, hemizygous transformed (T_0 = F1) plants will be tested by PCR or Southern blots for the presence of A1 ACCase sequences unique to the transformation vector, grown to maturity in growth chambers, greenhouse or field, and self-pollinated when possible, or backcrossed to a

10 nontransformed parent. F2 or backcross progeny are grown in the greenhouse and field and tested for Basta resistance and presence of the A1 ACCase transgene to identify homozygous transgenic plants. Homozygous inbred transgenic lines are then developed.

2.   Analysis of ACCase A1 transformants

15   Plants recovered from at least 100 independent transformation events (i.e., from different bombarded embryos) for both the UBI1 and GLB1 vectors are recovered. Regenerated plants are tested for the presence of the intact ACCase A1 transgene and its cosegregation with the Basta-resistance marker. Homozygous and heterozygous transgenic lines are assayed for total

20 ACCase activity in leaves for UBI1 transformants and in developing embryos (22-26 DAP) for both UBI1 and GLB1 transformants. Sethoxydim and haloxyfop inhibition are used to quickly determine the levels of herbicide-sensitive ACCase I (plastidic) and the herbicide-insensitive ACCase II activity in both leaves and embryos. Increased expression of the ACCase A1 transgene

25 contributes to plastidic ACCase activity and not to ACCase II activity. Kernel fatty acid and oil content are analyzed at maturity and their relationship with ACCase I determined by methods well known to the art.

Transformants that differ in kernel ACCase activity and/or fatty acid and oil content are then selected for more detailed analysis of embryos

30 throughout development (4-day intervals from 16 DAP to maturity). These analyses include RNAse protection assays to determine total A1 + A2 transcript levels using a non-specific probe and to determine relative levels of endogenous A1 versus transgene A1 transcripts by use of antisense riboprobes spanning the

5' UTR region of the A1 transgene constructs. Western blots of total proteins separated by SDS-PAGE gels are probed with the ACCase I-specific antibody described hereinabove or with avidin and analyzed by densitometry to distinguish changes in the 227-kD ACCase I and 219-kD ACCase II isoforms.

5   ACCase I activity, fatty acid and lipid content are determined in embryos at each stage of development. These analyses determine whether expression of an additional gene(s) for plastidic ACCase increases ACCase activity and consequently fatty acid and oil content in maize tissues, especially in embryos.

3.   Transformation with maize ACCase A1 antisense transformation vectors

10   Antisense transformation vectors were constructed by blunt-end ligation of nucleotides 1-833 of SEQ ID NO:5 in reverse orientation into multicloning sites of both the GLB1 and UBI1 plasmids. A sense construct with the same 833-bp cDNA sequence also was made with the GLB1 plasmid to serve as a transformation control. Insert orientations were verified by restriction

15   mapping. UBI1 antisense, GLB1 antisense, and GLB1 sense constructs were introduced into > 2100, > 2900 and > 2000 embryos, respectively, and Basta-resistant callus were selected.

If antisense expression results in significant reduction in ACCase activity, it may not be possible to obtain viable callus or plants from the

20   constitutive UBI1 antisense transformants. Similarly, plants transformed with the embryo-specific GLB1 antisense construct may exhibit deleterious effects on embryo development. Thus, failure to obtain transgenic progeny containing the antisense ACCase gene from these transformations may indicate that ACCase activity cannot be downregulated without loss of viability.

25 4.   Analysis of ACCase A1 antisense transformants

All Basta-resistant cultures will be regenerated. The presence of the UBI1 and GLB1 antisense constructs will be determined by PCR analysis for unique transgene sequences such as the *Ubi-1* intron/ACCase A1 junction or *Glb1* 5' UTR/ACCase A1 junction, or by Southern blotting to detect unique

30   fragments. Plants and lines homozygous or heterozygous for the antisense transgene are analyzed for steady state level of the ACCase A1 antisense transcripts in appropriate tissues/organs (such as leaves, tassels, ears, embryos and endosperm for UBI1; leaves and embryos for GLB1) by using ACCase A1

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sense riboprobes for hybridization on RNA blots or for RNAse protection assays.
Total ACCase activity (both ACCase I and ACCase II isoforms) and fatty acid
and lipid content are determined for the antisense transgenic lines and for
corresponding tissues from nontransformed control plants. These analyses show

5   whether ACCase A1 antisense transgenes are expressed in plants and, if so,
whether expression is associated with reduced ACCase activity and altered fatty
acid and lipid content in maize.

EXAMPLE XIII

Expression of Plastidic and Cytosolic ACCases during Plant Development

10   Intact embryos are isolated from developing kernels of field-
grown inbred B73 at 2 to 4 day intervals between 16-42 DAP and frozen
immediately in liquid nitrogen. Samples also are saved for fresh and dry weight
determinations. Subsamples from each stage are analyzed for total lipid and
fatty acid content. Seedling leaves are sampled along the leaf blade ranging

15   from the etiolated, meristematic basal region to the fully-expanded, green tip.
Leaves and other tissues (e.g., epidermis) of maize genotypes that accumulate
anthocyanin pigments are also analyzed to assess whether a specific ACCase
(such as a cytosolic ACCase) is more highly expressed in tissues in which
malonyl-CoA also is required as a substrate for chalcone synthase in the

20   flavonoid pathway leading to anthocyanin synthesis.

Gene-specific antisense riboprobes in RNAse protection assays
are employed to determine A1, A2, B1 and B2 transcript levels. The
corresponding sense transcripts are produced *in vitro* and used as standards to
verify specificity and quantitate the sample transcript levels. Quantitation is

25   done on an AMBIS radioanalytic image system. Herbicide inhibition of total
ACCase activity provides an assessment of levels of herbicide-sensitive ACCase
I (plastidic) and the herbicide-insensitive ACCase II activity in these tissues.
ACCase I and II isoforms are separated by ion-exchange chromatography. Total
proteins are separated by SDS-PAGE and Western blots probed with avidin to

30   detect the biotinylated 227-kD ACCase I and 219-kD ACCase II isoforms or
probed with ACCase I-specific antibodies.

EXAMPLE XIV

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Identification and Isolation of an Oat Gene Associated with Groat Oil Content

Cultivated oat, *Avena sativa* L. (2n=6x=42), is grown mostly in temperate regions. In many parts of the world, oat grain is produced for many

5   purposes such as for animal feed, human food, and cosmetic and pharmaceutical additives (Webster, 1986). Seventy-eight percent of the world oat production is used for livestock feed (Schrickel, 1986). Among the cereals, oat groats have the highest lipid concentration (Morrison, 1978), but due to their high fiber content oat is lower in energy value than many other cereals when used for livestock

10 feed. Oat is also unique among the cereals because more than 50% of total seed lipid is deposited in the starchy endosperm rather than the embryo of developing caryopses (Peterson and Wood, 1997; Youngs, 1986). An increase in oil content is desirable for making oat a higher-energy feed grain. On the other hand, a decrease in oil content would make oat a more attractive commodity for use in

15 many current human diets where oat is desired for the type and concentration of its fiber (Ripsin et al., 1992).

A polygenic pattern of inheritance has been indicated for groat oil content with heritability estimates ranging from 63 to 93% (Schipper and Frey, 1991a) with primarily additive gene action (Thro and Frey, 1985). The average

20 groat oil concentration in contemporary cultures is 6 to 7% but variation from 2 to 16% oil occurs in diverse germplasm (e.g., Kanota has about 11%, Ogle about 9% and Marion about 8% of oil (tryglycerides plus polar lipids) content, per dry weight). Genotype by environment interaction for this trait is small (Gullard, 1986; Branson and Frey, 1989). After five cycles of a recurrent selection

25 program, Schipper and Frey (1991b) were able to produce oat lines with groat oil content greater than 16%. This response to selection is similar to the classical Illinois long-term selection experiments for oil content in maize (*Zea mays* L.) that resulted in dramatic progress toward altering the oil content in the original variety (Dudley et al., 1974). These studies demonstrate that, except when

30 physiological limits are encountered, long-term selections for a quantitative, highly heritable and environmentally stable trait can prove successful. Analyses of 5987 oat samples representing a wide range of *Avena* germplasm showed a range of 2 to 12% for free non-polar lipids, with polar lipids contributing an

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additional 2 to 3% (Youngs et al., 1982). Combinations of alleles for changing
the groat oil content beyond this range may exist because this is mostly an
unselected trait that exhibits large phenotypic diversity. Therefore, genetic
dissection of this trait could expedite a program aimed at modifying the oil level
5   of oat.

The recent explosion in development of restriction fragment
length polymorphism (RFLP) linkage maps and their use in identifying genomic
regions which affect quantitatively inherited traits is well documented (Phillips
and Vasil, 1994). Molecular genetic studies relating to seed oil content have
10 been reported for major oil-producing crop plants such as rape seed (*Brassica
rapa* spp. *oleifera*; Tanhuanpaa et al., 1995a and 1995b), soybean (*Glycine max*
L., Diers et al., 1992; Mansur et al., 1993; Brummer et al., 1997) and maize
(Goldman et al., 1994; Alrefai et al., 1995). These studies indicate that genomic
regions can be identified which significantly influence seed oil content. In the
15 maize long-term Illinois selection material, two populations, the high oil by low
oil population (Alrefai et al., 1995) and the high protein by low protein
population (Goldman et al., 1994) were analyzed to identify genomic regions
influencing kernel oil content. In both populations a region on chromosome 6
was identified near the linoleic acid1 locus. In the one case, this region explains
20 63% of the phenotypic variation in the ratio of oleic to linoleic (18:1 to 18:2)
fatty acids and also influenced the total kernel oil concentration (Alrefai et al.,
1995). To date, oat plants with an altered fatty acid composition as a result of a
qualitative genetic mutation have not been identified, possibly due to the
hexaploid nature of the genome.

25   In monocots, graminicides cause plant cell death by inhibiting
ACCase and cessation of fatty acid biosynthesis (Burton et al., 1989). Maize
mutants have been isolated that grow in the presence of normally lethal
concentrations of ACCase-inhibiting herbicides, exhibit up to 20-fold increase in
wild-type ACCase activity, and synthesize fatty acids in the presence of
30 herbicide (Parker et al., 1990). Other results point to the role of ACCase in
regulating seed oil deposition. A herbicide-insensitive ACCase mutation, Acc1,
exhibits increased ACCase activity in corn embryos paralleling the reporting
time course of oil deposition in this tissue (Somers et al., 1993). In developing

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seeds of caster bean and rape, measured activity of ACCase correlates well with
lipid accumulation providing additional evidence as to the role of this enzyme in
fatty acid biosynthesis (Simcox et al., 1979; Turnham and Northcote, 1983).
Moreover, expression of *Arabidopsis* cytosolic ACCase in *B. napus* plastids

5   alters fatty acid composition, with the largest effect being an increase in oleic
acid, and increasing total seed oil content by about 5% (Roesler et al., 1997).
This increase in oleic acid is consistent with studies in maize indicating a
positive correlation between percentage of oleic acid and total seed oil content
(Alrefai et al., 1995).

10   Materials and Methods
Genetic Material

Two oat $F_{2:6}$-derived recombinant inbred populations developed
by single seed descent (SSD) were used in this study: a population of 137
recombinant inbred lines (RILs) developed from the cross of a facultative winter-

15   type cultivar, Kanota, by a spring cultivar, Ogle, (KO) and a second population
of 139 RILs from the cross of Kanota by another spring cultivar, Marion, (KM).
A portion (71 RILs) of the first population had been employed in previously
reported oat mapping and QTL studies (O'Donoughue et al., 1995;
Siripoonwiwat et al., 1996; Holland et al., 1997). The second population was

20   chosen on the basis of having a common parent, Kanota, to provide a degree of
"biological replication". These materials were grown in 1992-1995 in a
randomized complete block design at the following locations;

Kanota X Ogle (KO):   Aberdeen, Idaho, 1992 and 1993; Ithaca, New
                           York, 1993; and St. Paul and Rosemount,

25   Minnesota, 1994.

Kanota X Marion (KM):   St. Paul and Rosemount, Minnesota, 1994; and St.
                           Paul, Minnesota, 1995.

At Aberdeen, Idaho, the materials were grown in 3 replicates of four-row plots of
2.5 m in length, 30 cm between rows with the center 2 rows harvested. At

30   Ithaca, New York, the materials were grown in 3 replicates of six-row plots of 4
m length and 18 cm between rows. At St. Paul and Rosemount, Minnesota, the
materials were grown in 4 replicates of hill plots, with 30 seeds planted per hill

on 30 cm grid spacing. The exact parents used to generate the populations were included as single or multiple entries in each replicate.

Oil Content Analysis

To reduce the number of samples needed for analysis, equal

5   amounts of sample from each replicate at a given location/year were bulked to represent a single balanced sampling per line. This resulted in five measurements for each RIL in the Kanota X Ogle population and three for the Kanota X Marion population.

Groat oil content was measured by a Bran+Luebbe InfraAlyzer

10   500 on dehulled ground samples. The Near Infrared (NIR) calibration was based on total fat as determined by acid hydrolysis. The calibration set contained 348 oat samples (representing commercial and elite breeding lines from North American oat breeding programs) with fat contents that ranged from 4.95 to 14.01%. The calibration equation had a correlation coefficient of 0.985, a

15   Standard Error of Estimate of 0.299, and a F-value of 11500. Acid hydrolysis extracts the total lipid content of the groat. Thus, the reported values represent the sum of the neutral lipids (triglycerides) plus the polar lipid fractions (Youngs et al., 1982). Thus, these values are higher than those obtained from ether extracts which only contain the neutral lipid fraction.

20   Oat ACCase Clones or PCR Products

An oat groat cDNA clone was isolated based on its hybridization to a maize cDNA fragment which included portions of the less conserved mid-peptide sequence and the transcarboxylase domain (Shorrosh et al., 1994). Sequence identity indicated that the oat cDNA (3031 bp) corresponded to maize

25   ACCase nt 3124-6169 (Egli et al., 1995). The 1010-aa predicted polypeptide was nearly identical to portions of wheat (89%) and maize (84%) plastidic multifunctional ACCases; it had lower identity to cytosolic ACCases, including wheat (67.5%) and alfalfa (64.6%) (Gornicki et al., 1994; Shorrosh et al., 1994).

An amplified 'Prairie' oat developing groat cDNA library (R.

30   Skadsen, USDA, Madison, WI) cloned into the EcoRI site of λZAPII (Stratagene) was screened with a [^{32}P-dCTP]-labelled maize ACCase cDNA probe (Ausabel et al., 1997) derived from an EcoRI digest of clone 18-5 (nt 2020-5927; Egli et al., 1995). Plaques from eleven partially purified positives

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were eluted in SM medium (Sambrook et al., 1989) and excised *in vivo* to obtain

pBlueScript plasmids carrying oat cDNA inserts, according to the

manufacturer's instructions (Stratagene Corp.). Five colonies per transformation

reaction were screened (as colony lifts) with an AflII-BbrPI fragment of maize

5   ACCase cDNA (4281-5797; Egli et al., 1995). One clone, oat-91, hybridized to

the probe. Oat-91 was sequenced using the ABI Prizm system (Perkin-Elmer

Corp.) at the Advanced Genetic Analysis Center (Univ. of Minnesota, St. Paul,

MN). Sequence identity indicated the oat-91 EcoRI insert corresponded to

maize ACCase nt 3124-6169 (Egli et al., 1995). Oat ACCase cDNA sequence

10   was compared to other plant ACCases with the BESTFIT program (BESTFIT,

Wisconsin Package V. 9.1, Genetics Computer Group (GCG), Madison, WI).

         For PCR, Pileup alignments of maize, wheat, barley, and rice

ACCase coding sequence (GCG, Madison, WI; 12, 18, F, R) were used to

identify conserved regions (i) for design of new primers or (ii) regions of identity

15   to available maize ACCase oligos, to be used for PCR amplification of oat

ACCase. Two maize primers, PCR2 (5'-ggcagagcaa agcttggagg; SEQ ID

NO:25) and PCR1 (5'-tccaccagag aagcctctcc; SEQ ID NO:26) appeared like to

amplify oat cDNA based on their identity to other monocots and previous use to

amplify soybean ACCase. These primers are located within the transcarboxylase

20   domain, surrounding the acetyl-CoA binding site of ACCase. An amplified

'Prairie' oat seedling leaf cDNA library cloned into the EcoRI site of IZAPII

(Skadsen, University of Wisconsin) was used as a source of cDNA. One I of the

oat cDNA library was amplified with maize primers (PCR2/PCR1), using the

Expand High Fidelity PCR system (BMB) in a GeneAmp PCR System 9600

25   thermal cycler (Perkin-Elmer) after denaturing phage heads at 94°C for 10

minutes. The single, 222-bp product was purified by Wizard PCR Plus

(Promega Corp.) and sequenced in an ABI377 (Perkin-Elmer) using PCR1 as

primer. The resulting sequence appeared to be from oat since it had high identity

to maize plastidic ACCase (89%). Additional 5' cDNA was obtained by (i) PCR

30   of the oat cDNA library with T3/PCR1 primers to generate a complex band

mixture and (ii) reamplification of 0.1 I products with maize ACCase primers

1324+ (5'-caatcacgtcagactg; SEQ ID NO:27)/PCR1 to obtain a 1.9-kb product

with the same 3' sequence as the PCR2/PCR1 product.

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RFLP and Statistical Analysis

　　　　　　Briefly, two statistical procedures were used to determine the
location, effect and genotype by environment interaction (GxE) for significant
QTLs. The first procedure was single factor ANOVA (ANOVA and GLM

5　procedures; SAS Institute, 1990) with the added criteria for consistency in
association of genomic regions with a trait over environments and linkage
distances (Kianian et al., 1998). If the association were significant when
considering the average of all the environments, the association was also
significant over the majority of the environments (3 of 5 in KO and 2 of 3 in

10　KM), and closely linked markers within that region, if available, also showed
significant associations. A "backward elimination" process as described by
Kennard and Havey (1995) was used to construct a multi-locus model containing
the most important markers identified by the ANOVA procedure. Marker loci
were excluded one at a time from this model, based on the criterion of least

15　significant (P < 0.05) Type-III sum of squares (Kennard et al., 1994). In
addition, MQTL software (Tinker and Mather, 1995a & b) was used to
strengthen and validate the ANOVA results. This software performs Simple
Interval Mapping (SIM) and Simplified Composite Interval Mapping (sCIM). It
also has the advantages of searching for one QTL while simultaneously

20　accounting for the effect of others, providing separate analyses for each
environment, and calculation of a threshold to control the type-I error rate. One
thousand permutations were performed to estimate the threshold for a type-I
error rate below 5%. Significant QTL regions were determined as described by
Tinker et al., 1996. These regions were grouped in a multi-locus linear model to

25　estimate the overall phenotypic variance explained by the model. However,
additional criteria were used to include markers in the final MQTL model, which
gave 10 cM spacing between QTLs, and minimized the number of regions
excluding those markers explaining less than 5% of the phenotypic variance,
while maintaining most of the overall R^2.

Results

Trait Data

Groat oil concentration for both populations showed a relatively
continuous distribution with many of the individuals having values within one

5   standard deviation of the parents (Table VI). Among the three parents, Kanota
showed the highest concentration of groat oil content followed by Marion and
then Ogle. The groat oil content value for the Kanota line derived from the
parent used in generating the KM population was higher than that used for the
KO population (Table VI). This is not unexpected: Kanota is a heterogeneous

10   population that shows variation for other traits, but individual parent lines were
used in population development and were maintained as controls for each
population. Large numbers of individuals having values outside the range of
parents were observed for both populations (Table VI). Transgressive segregants
tended more toward the lower groat oil concentration than the high. The KO

15   population contained individuals with the highest and lowest groat oil
concentrations, possibly due to a larger difference in parental values (3%
between Kanota and Ogle, 2% between Kanota and Marion). Overall, the KM
population contained more transgressive segregants than the KO population
(Table VI).

20

Table VI. Groat oil concentration and percent transgressive segregants in two recombinant inbred oat populations

Population (P1 x P2)	Location	Parents		Population				% Transgressive segregants			
		P1	P2	Mean	SD	Min.	Max.	X<P2	X>P1	X<P2-SD	X>P1+SD
Kanota X Ogle	Aberdeen 92	11.5	8.3	9.7	1.3	7.5	14.0	13	8	0	2
	Aberdeen 93	10.5	8.4	10.1	1.6	7.2	15.4	18	35	0	8
	Ithaca 93	10.9	7.1	9.0	1.4	6.5	13.6	8	8	0	2
	St. Paul 94	10.9	7.7	9.2	1.4	6.6	13.4	13	10	0	2
	Rosemount 94	11.1	8.4	9.0	1.3	6.7	12.6	30	5	5	1
	Average	11.0	8.0	9.4	1.4	6.9	13.8	16	13	1	3
Kanota X Marion	St. Paul 94	12.9	10.2	10.2	1.0	8.0	13.3	47	1	12	0
	Rosemount 94	12.2[a]	9.2	10.4	1.2	7.5	14.2	10	7	1	3
	St. Paul 95	10.0	8.4	8.8	0.7	7.1	10.8	26	2	4	1
	Average	11.5	9.3	9.8	1.0	7.5	12.8	28	3	6	1

[a] Adjusted average value for the parents calculated based on the mean of population at that location divided by the overall population mean times the average parental values was used in this calculation (i.e., (10.4/9.8)11.5).

SD = Standard Deviation

Table VII. Phenotypic correlations for groat oil concentration between environments*

Population	Location	Aberdeen 92	Aberdeen 93	Ithaca 93	St. Paul 94
Kanota X Ogle	Aberdeen 93	0.94			
	Ithaca 93	0.94	0.97		
	St. Paul 94	0.90	0.91	0.92	
	Rosemount 94	0.91	0.95	0.96	0.94

Population	Location	St. Paul 94	Rosemount 94
Kanota X Marion	Rosemount 94	0.87	
	St. Paul 95	0.80	0.71

* All values are considered significant at $P < 0.0001$.

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The correlation among the different environments for oil content was high (Table VII). Measurements for the KO population had an average R of 0.93 with the lowest value of 0.90 between material grown at Aberdeen, ID 1992 and St. Paul, MN 1994 (P < 0.0001). Values for the KM population were

5 less well correlated (average R of 0.79) with the lowest correlation between the samples grown in Rosemount, MN 1994 and St. Paul, MN 1995 (P < 0.0001). The difference in the size of the population correlations could be due to a smaller range of values for the KM population (5%) as opposed to the KO population (7%), or fewer measurements (3 vs. 5 environments), or both.

10 Kanota X Ogle Population

RFLP Data

A hexaploid oat RFLP map that contains 561 loci has been generated from 71 RILs in the KO population (O'Donoughue et al., 1995). Initial quantitative analysis was performed using this subset of the KO

15 population, the available trait data, and 360 of the 561 loci that are co-dominantly segregating and evenly distributed across the oat genome. To take advantage of the entire population and to add power to the analysis, the remaining 66 RILs in this population were also mapped. To date, 153 loci have been mapped with these individuals. Detection of QTL with either the subset

20 (360 markers and 71 individuals) or the entire data set (153 markers and 137 individuals) of the KO population did not make a major difference, except in two situations. In both cases, QTLs were detected in the subset, but were not detected in the entire population due to the absence of RFLP data for the remaining 66 RILs. Results of the entire population are presented herein except

25 for the aforementioned differences.

An RFLP map containing all 137 individuals and the 153 mapped loci was generated and was in accord with the published map (O'Donoughue et al., 1995) except for linkage group 3. An inter-varietal translocation is believed to involve this linkage group. In the final analysis, the published map was used

30 for this region. Additional information from aneuploid analysis (Kianian et al., 1997) was also taken into account when two linkage groups were assigned to the same chromosome. Thus, linkage groups 5 and 7 assigned to chromosome 5C, linkage groups 6 and 20 assigned to chromosome 16, and linkage groups 4 and

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12 assigned to chromosome 21, were analyzed as three distinct units. In order to have a complete coverage of the genome for SIM and sCIM, 344 mapped loci (191 loci scored on only 71 RILs) were used in the MQTL analysis.

Quantitative Analysis

5   Analysis of variance using the criteria mentioned revealed eight linkage groups and one unlinked marker significantly associated with groat oil content ($P < 0.05$ for individual environment and $P < 0.0001$ using means across environments). These regions are on linkage groups 6 and 7 (Table VIII). Two additional genomic segments on linkage group 37 and an unlinked locus

10   Coleoptal were significant in tests of the first 71 individuals of the mapping population but could not be verified on the whole population due to a lack of RFLP data for the entire set. The positive alleles (those that cause an increase in groat oil content) for all of the genomic regions influencing this trait were from the Kanota parent (Table 3). Overall, the alleles from Kanota contributed 2.8%

15   (4.1% if linkage group 37 and Coleoptal loci were included) to the groat oil content. Regions on linkage groups 6 and 11 are considered highly significant and remained in the full model after the backward elimination process (Table 4). However, the two additional loci from linkage group 37 and Coleoptal were also included in the model if only the first 71 individuals in this population were

20   being analyzed. However, the two additional loci from linkage groups 37 and Coleoptal were also included in the model if only the first 71 individuals in this population were being analyzed. Locus Xcdo665B on linkage group 11 accounted for 36% to 48% (depending on the environment) of the phenotypic variance for this trait ($P < 0.0001$). For these linked loci, a substitution of

25   Kanota alleles for their Ogle counterparts caused an average increase of 2% in groat oil content. Substitution of Kanota alleles for Ogle alleles at the locus Xcdo1414B (or Xumn51B) on linkage group 37 caused an average increase of 1% in groat oil content and was the second most significant locus considering only the first 71 individuals in this population.

Table VIII. Genomic regions significantly influencing groat oil content in oil identified by single factor analysis of variance

	Kanota X Ogle					Kanota X Marion			
Linkage Group	Locus[a]	Source	R^2	% Oil[b]		Linkage Group	Locus[a]	Source	R^2	% Oil[b]
6	Xcdo1357C	Kanota	0.10	0.8		3	Xbcd1562A	Kanota	0.05	0.4
11	Xcdo665B	Kanota	0.38	2.0		11	Xcdo665B	Kanota	0.18	0.7
37[c]	Xcdo1414B	Kanota	0.10	1.0		22	Xumn5004	Kanota	0.05	0.3
Unlinked[c]	Coleoptal	Kanota	0.13	0.3		5X[d]	Xcdo1199 C	Kanota	0.11	0.7
Total				2.8 (4.1[b])		Total				2.1

a Locus with the highest R^2.

b Substitution of the source alleles will cause on average (based on average of values from each of five environments in KO and three in KM) this change in groat oil concentration. $P < 0.0001$ for the mean across all environments and $P < 0.05$ for each individual environment.

c Significant region identified only in the first 71 individuals of this population.

d More markers are needed to accurately place this linkage group on the hexaploid oat RFLP map.

Table IX. Multi-locus models for groat oil concentration in oat

Population	ANOVA Model[a]	MQTL Model[b]
Kanota X Ogle	*Xcdo1357C* (6, K)	*Xcdo665B* (11, K)[c]
	Kcdo665B (11, K)[c]	*Xcdo1414B* (37, K)
	Xcdo1414B (37, K)[d]	*Coleoptal* (Unlinked, K)
	Coleoptal (Unlinked, K)[d]	
Total R²	0.45 (0.67)[e]	0.47
Kanota X Marion	*Xcdo665B* (11, K)[c]	*Xcdo665B* (11, K)[c]
	Xumn5004 (22, K)	*Xumn5004* (22, K)
	Xcdo1199C (5X, K)	*Xcdo1199C* (5X, K)
Total R²	0.28	0.20

[a]   The linkage group and parent contributing the positive allele are depicted in parentheses (i.e., linkage group 11 with Kanota allele being positive (11, K)). The models are based on five environments in KO and three in KM.

[b]   The VM/VP (variance explained by the QTL(s) over the phenotypic variance) is presented as the R² for MQTL.

[c]   *XaccaseA* is closely linked to *Xcdo665B* and gives the same relative effects as *Xcdo665B*.

[d]   These additional loci fit the model when the analysis included only the first 71 individuals in this population.

[e]   Total R² with markers marked d is presented in parentheses.

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Quantitative analysis using the MQTL software gave a slightly different result. Like the ANOVA results, a highly significant locus (P < 0.0001), Xcdo665B on linkage group 11, was identified at a test statistic (TS) value of 290.2 that is roughly equal to a LOD score of 64 (0.22 × TS). This

5   locus overshadowed any other significant region and accounted for more than 37% of the phenotypic variance [Variance QTL Main effect (VM)/Variance Phenotypic (VP) = 38%, Variance QTL × environment Interaction and VM (VI)/VP = 38% and Variance Genetic from background markers (VG)/VP = 7%)] and a change of 2% in groat oil content (Figure 23). However, secondary

10   loci that showed significance below the threshold value (threshold TS = 58.2 after 1000 permutations) were inferred when either SIM or sCIM gave evidence for QTLs. Xumn51B on linkage group 37 and the Coleoptal locus, which is unlinked, were significant at test statistic values of 49.9 and 44.8 respectively. The three loci together accounted for 47% of the phenotypic variance and a

15   change of 3.3% in groat oil content (VM/VP = 47%, VI/VP = 48% and VG/VP = 7%). For all three loci the Kanota parent contributed the positive allele. These loci showed a remarkable consistency over the five environments. Thus, there were no significant peaks inferred from the QTL × E interaction scan (Figure 23).

20   Kanota X Marion Population
     RFLP Data

The results from an initial quantitative analysis of the KO population (first 71 RILs) for groat physical and chemical traits were used to determine markers for mapping the KM population. The markers mapped were

25   not chosen for their association with groat oil content in the KO subset population, but for their association with other groat chemical and physical characteristics (Kianian et al., 1998). Since the level of polymorphism in hexaploid oat is relatively high, most probes detected sequences mapping to more than one location. A total of 60 loci were mapped with the 139 RILs in the

30   KM population. These markers mapped to 20 of the 38 linkage groups that had been identified in the KO population (linkage groups 1, 2, 4, 5, 8, 9, 18, 19, 21, 24, 25, 26, 30, 33, 34, 35, 36, 37, 38 were not covered). The linkage groups not covered were, in general, the smaller ones consisting of only two to a few

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markers in the KO map. The data from the KM population generated an additional linkage group of two loci, and 13 markers remained unlinked. Except for linkage group 37 and *Coleoptal* locus, this KM map covered all regions with significant associations with groat oil concentration in the KO population.

5   Quantitative Analysis

Single factor ANOVA revealed four linkage groups significantly associated with groat oil content in the KM population. These were groups 3, 11, 22, and 5X (P < 0.05 for individual environment and P < 0.0001 using means across environments). As in KO, all of the genomic regions influencing this trait

10   were from the Kanota parent. Overall, the Kanota genomic regions contributed 2.1% to the groat oil content. Regions on linkage groups 11, 22, and 5X (group 5X has been tentatively assigned to linkage group 5 but more markers are needed to accurately place this linkage group on the hexaploid oat RFLP map) are considered highly significant and remained in the full model after the backward

15   elimination process. As in the KO population, locus *Xcdo665B* on linkage group 11 was highly significant for its effect on groat oil content (P < 0.0001), accounting for 16% to 20% (depending on the environment) of the phenotypic variance for this trait. Overall, the three significant loci explained 15% of the phenotypic variance and together accounted for a total difference of 1.7% in

20   groat oil content.

Analysis by SIM or sCIM gave the same result as ANOVA. Linkage groups 11, 22, and 5X were significant in the KM population. Locus *Xcdo665B* on linkage group 11 was significant at a test statistic value of 70.5 (LOD = 15.5). This locus was not as dominant in the KM population as it was in

25   the KO population. This locus alone explained 18% of the phenotypic variance (VM/VP = 18%, VI/VP = 19% and VG/VP = 3%) and accounted for 0.9% change in groat oil content. The other significant locus above the threshold value was *Xcdo1199C* on linkage group 5X (TS = 33.1) accounting for a change of 0.2% in groat oil concentration. A secondary locus inferred was *Xcdo506B* on

30   linkage group 22, which accounted for 0.4% change in oil content. These three loci together accounted for 20% of the phenotypic variance and 1.5% in groat oil content (VM/VP = 20%, VI/VP = 22% and VG/VP = 3%). As expected from the trait data, these loci showed a varied effect over the three environments and one

interaction on linkage group 11 is notable (Figure 23). The QTL × E interaction

peak on linkage group 11 was at TS = 6.6, slightly below the significant

threshold, and was due to a range of 0.5% in groat oil content explained [the

values for the groat oil content were 0.6% (St. Paul, Minnesota, 1995) and 1.1%

5   (Rosemount, Minnesota, 1994)].

ACCase and its Relationship to Groat Oil Content QTLs

        An oat groat cDNA was isolated based on its hybridization to a

maize cDNA fragment which included portions of the less conserved mid-

peptide sequence and the transcarboxylase domain (Shorrosh et al., 1994). The

10   3031-bp oat cDNA was nearly identical to wheat (90%) and maize (84%) genes

encoding plastidic ACCase (Egli et al., 1995; Gornick et al., 1997). A 424

nucleotide sequence form the 3' end of the insert was translated and compared to

other plant ACCase peptides. The oat peptide sequence was 93% identical to

plastidic isozymes from maize and wheat (Egli et al., 1995; Gornicki et al.,

15   1997), but only 78 to 83% identical to cytosolic ACCase from maize, wheat

(Gronicki et al., 1994), or alfalfa (Shorrosh et al., 1994).

        The 3031-bp oat ACCase cDNA clone identified a single

polymorphic locus in the KO population (AccaseA) and two polymorphic loci in

the KM population (AccaseA and AccaseB). The AccaseA locus maps 2.3 cM

20   from Xcdo665B and 3.7 cM from Xisu2287 on linkage group 11. The AccaseB

locus maps 11 cM from Xcdo665D and 7.3 cM from Xbcd1729B. In the KO

population AccaseA explains on average 37% of the phenotypic variance and in

KM 18% of the phenotypic variance for groat oil content (for both cases P <

0.0001 using means across all environments). The individuals in both

25   populations can be divided into two groups based on their genotype for this locus

and groat oil content using the mean across all environments (Figure 3). These

two classes differ in their oil content by an average of 1.67% in KO and 0.78%

in KM. Results of QTL analysis for the AccaseA locus are the same as that

reported for Xcdo665B (Table 4).

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Table X. Peptide sequence identity of oat and other multifunctional ACCases.
Partial, internal sequences are 1010 amino acids unless noted otherwise.
Presence of an N-terminal plastid transit peptide is noted, if known.

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ACCase Gene source	GenBank #	Transit Peptide?	% identity
Avena sativa	AF072737	?	100
Triticum aestivum	AF029895	yes	89.2
Zea mays	U19183	yes	84.1
Triticum aestivum	U10187	no	67.5
Zea mays	Egli, unpubl.	?	78.6 #
Hordeum vulgare	X99102	?	71.9 §
Arabidopsis thaliana	L27074	no	63.7
Brassica napus	X77576	yes	63.9

based on 145 aa at the C-terminus of oat-91
§   based on 498 aa at the C-terminus of oat-91
?   partial cDNA, unknown N-terminus

Discussion

The ACCase cDNA clone from developing oat seeds hybridizes to a genomic region that is linked to a QTL locus on linkage group 11 having a major influence on the total lipid content of oat groats. The influence of this locus was detected in various environments and two genetic backgrounds, thus confirming the value of biological as well as environmental replication. These results provide strong support for the hypothesis that ACCase has a major role in determining groat oil content in crop plants. Either allelic variation in ACCase activity controls oil content in oat groats, or a gene(s) closely linked to ACCase is responsible. In other studies of oat directed at transferring a major crown rust resistance locus on linkage group 11 (Bush et al., 1994), lines with the introduced rust resistance gene often had altered groat oil content. These results further indicate the likely presence on linkage group 11 of a genetic locus with a critical role in the fatty acid biosynthetic pathway in oat. Mutations, which cause a gradual but demonstrable major effect on a biochemical pathway, are believed to be the most common type in polyploids (Sears, 1972).

Identification of a major quantitative trait locus influencing the biochemical composition of grain is not unusual. Studies of fatty acid, starch, and protein concentrations and composition provide good examples (Goldman et al., 1993 and 1994; Alrefai et al., 1995; Hu et al., 1995). In many of these cases,

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the significant region is in close proximity to a known genetic locus with a demonstrated effect on the trait of interest. For instance, in maize, a major genomic region for fatty acid concentration and composition has been identified near the *linoleic acid-1* locus, and one influencing protein and starch

5   concentration was identified near the *Shrunken-2* locus (Goldman et al., 1993 and 1994; Alrefai et al., 1995). These and numerous other reports bolster the hypothesis that allelic variants at known genetic loci may be responsible for quantitative effects (Robertson, 1985).

          Reliable identification of genomic regions with an influence on a

10   particular quantitative trait has always been difficult. In a number of studies conducted on a given quantitatively inherited trait in various populations and environments, the most significant regions tended to be in common (Goldman et al., 1994; Alrefai et al., 1995). However, the accuracy of identifying a quantitative trait locus will depend, in part on the degree to which it influences

15   the trait in a given population. This is demonstrated in our study by the locus on linkage group 11 which has similar but varied effects in different populations. Marion has a higher concentration of groat oil than Ogle and more loci with positive influence on this trait. The locus on linkage group 11 increased the groat oil content to a lesser degree in the KM population than in the KO

20   population. Correspondingly, the relative proportion of variance due to the environmental influence on this locus was much higher in the KM population than in the KO population. This example illustrates the power of biological replication (i.e., having populations with a common parent) in identifying quantitative trait loci.

25   Identification of the most significant locus does not preclude the identification of other less important loci due to the influence of genetic background and environment on the overall effect. The consistency in identification of significant regions by various quantitative analysis methods in the KM population is a good example. The regions on linkage groups 5, 11 and

30   22 and Xcdo395C locus are considered to make important contributions to the groat oil content in this population. The Xcdo1199C locus located near the Xacor242 locus on linkage group 5 of the published hexaploid oat RFLP map (O'Donoughue et al., 1995) has as great an effect as Xcdo665B-XaccaseA region

on linkage group 11 in this population. This locus was not detected in the KO

population presumably because the Ogle allele had a similar influence as Kanota

and in the KM population the Marion parent was carrying the allele with positive

influence on the groat oil content. The other two significant loci in the KM

5   population have a relatively minor effect on this trait and would be more difficult

to detect in a population segregating for regions with a larger influence on oil

such as in the KO population.

Results from other QTL studies can help identify possible

pleiotropic effects of other traits such as heading date and vernalization response

10   on groat oil concentration. A region on linkage group 3 with alleles being

contributed by Kanota is implicated as having a major influence on heading date

and possibly yield, thus reducing its importance as having a direct effect on groat

oil concentration (Siripoonwiwat et al., 1996; Holland et al., 1997). The cultivar

Ogle was found to have loci on linkage groups 22 and 37 that influence plant

15   height in the KO population (Siripoonwiwat et al., 1996). Loci on linkage group

11 is believed to have an influence on test weight and other groat physical

characteristics, thus providing additional evidence of their role in oat groat

morphophysiology (Siripoonwiwat et al., 1996; Cakir et al., 1996). These and

other studies of quantitative traits in oat are helping identify genomic regions

20   that influence the growth, development and physiology of grain, the primary

product. Thus, genetic and molecular manipulation of the oat genome to

improve grain quality and quantity can be directed at critical segments.

Cultivated oat is a hexaploid composed of three ancestral

genomes designated A, C, and D (Rajhathy and Thomas, 1974). Recent studies

25   have indicated that the organization of hexaploid oat genomes into discrete

homoeologous groupings such as that of hexaploid wheat is difficult (Rooney et

al., 1994; O'Donoughue et al., 1995; Kianian et al., 1997). However, C-genome

chromosomes, due to their distinctiveness, have been used as a basis to identify

homoeologous segments among the other chromosomes (Kianian et al., 1997).

30   Thus, it is believed that a type of segmental homology instead of whole

chromosome homology best describes the genome organization in hexaploid oat

(Kianian et al., 1997). Association of homoeologous genomic segments with a

quantitative trait further substantiates that relationship.

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The region marked by the probe CDO1414 can identify both the significant region on linkage group 11 and the one on linkage group 37 indicating possible homoeology of these regions. The same is true for probes CDO1199 for linkage groups 6 and 5X and CDO708 for linkage groups 6 and

5   22. Thus, the association of groat oil content with RFLP markers in oat is explained by at least three sets of homoeologous genomic segments.

In hexaploid wheat plastidic ACCase maps to the short arm of homoeologous group 2 near the telomere (Gornicki et al., 1997). Plastidic ACCase genes in wheat have been shown to be transcriptionally active in

10   seedling leaves but their effect on seed lipid content are unknown (Gornicki et al., 1997). ACCase in maize has been mapped to short arm of chromosome 2 near the centromere and long arm of chromosome 10 (Egli et al., 1995; Van Dee, 1994). A QTL with moderate effect on oil concentration in progeny of Illinois High and Low oil maize lines was located near the ACCase locus on maize

15   chromosome 2 (Berke and Rocheford, 1995). Additionally, there are numerous *defective kernel (dek), reduced endosperm (ren)*, and *Endosperm factor (Ef)* loci influencing grain morphophysiology located on chromosomes 2 and 10 of maize near these synthetic segments (MaizeDB). In maize, oil is stored in embryos and embryo size is associated with oil content of whole seeds (Dudley, 1974).

20   Unlike maize, oat grains contain significant amounts of lipid in the endosperm (Peterson and Wood, 1997), and thus QTLs affecting pathway flux could be more important than those altering seed anatomy. The regions in wheat and maize agree well in terms of conserved syntony and correspond to chromosome 4 of rice, linkage group C of diploid oat and linkage group 11 of hexaploid oat

25   map (Ahn and Tanksley, 1993; Van Deynze et al., 1995). This comparative evidence from other grass species and homoeologous evidence from oat further help reinforce the validity of the association of these genomic regions with groat oil content and composition in oat.

30   Summary

Oat groats are unique among cereals for the high level and the embryo-plus-endosperm localization of lipids. Genetic manipulation of groat quality traits such as oil is desired for optimizing the value of oat in human and

livestock diets. A locus having a major effect on oil content in oat (*Avena sativa* L.) groats was located on linkage group 11 by single factor analysis of variance, simple interval mapping and simplified composite interval mapping. A partial oat acetyl-CoA carboxylase (ACCase) cDNA clone with >84% identity to genes

5   for plastidic ACCase of maize and wheat, which catalyzes the first committed step in *de novo* fatty acid synthesis, identified a polymorphism linked to this major locus. Similar QTL and ACCase locus placements were obtained with two recombinant inbred populations having one parent in common (Kanota X Ogle and Kanota X Marion) and containing 137 and 139 individual lines,

10 respectively. By having a common parent, these populations provide biological replication of the results in that significant genomic regions should be evident in analysis of multiple cross combinations.

Similar QTL and ACCase placements were obtained with two recombinant inbred populations having one parent in common (Kanota X Ogle

15 and Kanota X Marion) and containing 137 and 139 individual lines, respectively. By having a common parent, these populations provide biological replication of the results in that significant genomic regions should be evident in analysis of multiple cross combinations. These populations were mapped with RFLP loci (150 and 60) distributed over the genome and were grown over diverse locations

20 (4 and 2) and years (3 and 2) to provide replication for measurements of groat oil content. The locus on linkage group 11 linked to *AccaseA* accounted for up to 48% of the phenotypic variance for groat oil content. Additional QTLs in both populations were identified which accounted for an additional 10% of the phenotypic variance. Thus, the major locus plus additional QTLs represent three

25 homologous genomic segments in oat that can be followed by as few as three RFLP markers to produce a large shift in the groat oil content.

In contrast to oat, a maize ACCase gene was linked only to a moderate oil QTL. Unlike maize, where embryo size affects seed lipid concentration, oat stores significant amounts of lipid in the endosperm, thus

30 QTLs affecting pathway flux may be more important than those altering seed anatomy. If an ACCase gene is indeed the major groat oil QTL, differential ACCase gene expression and/or enzyme activity may control groat oil content.

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Moreover, if an ACCase DNA is a QTL in oat, it may be useful as a marker for
marker-assisted breeding in oat and other cereals, e.g., maize and wheat.

Table XI. Genomic regions significantly influencing groat oil content in oat.
5   Groat oil values were averages of five (KXO) or three (KxM) environments.

Kanota x Ogle

Locus	Linkage Group	High-Oil Parent	Coefficient of variation	Groat oil, % dry wt
Xcdo665B	11	K	0.38	2.0 #
XaccaseA	11	K	0.38	2.0
Coleoptal	unlinked	K	0.13	0.3
Xcdo1414B	37	K	0.10	1.0
Xcdo1357C	6	K	0.10	0.8
				4.1 Total

Kanota x Marion

Locus	Linkage Group	High-Oil Parent	Coefficient of variation	Groat oil, % dry wt
Xcdo665B	11	K	0.18	0.70 #
XaccaseA	11	K	0.18	0.70
Xcdo1199C	5X	K	0.11	0.70
Xbcd1562A	3	K	0.05	0.40
				1.80 Total

Xcdo665B and *XaccaseA* were associated with the same QTL.
Groat oil values were averages of five (KxO) or three (KxM) environments.
QTLs were identified by single factor analysis of variance.

                              EXAMPLE XV

                  Method to Decrease Oil Content in Oat Seed

                  Youngs (1986) reviewed 11 studies reporting lipid content of oat
groats and found an overall range of 2.0 to 11.8% oil concentration in oat groats.
Contemporary oat cultivars exhibit an average of 6 to 7% oil (1994-96 Midwest
Regional Uniform Nursery data). Recently, Peterson and Wood (1997) reported
on the composition and structure of high-oil oat selections ranging from 6.9 to
18.1% oil. While this level of lipid is beneficial for animal feeding because high

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oil is associated with higher energy value in the feed, high oil content in oats

causes rancidity problems during processing. Rapid liberation of fatty acids

from triglycerides by endogenous lipases results in fatty acid oxidation.

Furthermore, use of current oat cultivars results in relatively high-fat food

5   products, e.g., oatmeal. Oat processors indicate that a 50% reduction of groat

lipid content, while maintaining the superior agronomic characteristics of the

currently existing cultivars, would be highly desirable.

Oat is unique among the cereals in regard to seed lipid deposition

(Welch, 1995; Youngs, 1986). Most other cereals accumulate storage lipid

10   primarily in the embryo of developing caryopses. Mature oat groats contain

more than 50% of total seed lipid content in the starchy endosperm (Peterson and

Wood, 1997). Additionally, significant amounts of oil are found in the bran

fraction of processed oats. A substantial portion of the bran is in starchy

endosperm and aleurone cell layers which do not separate cleanly from the bran

15   as in wheat. Together the oat endosperm and bran fractions contain >90% of the

total lipid in the groat. Thus, the endosperm is the major source of the oil in the

groat.

Two distinct types of ACCase are known. The first type is typical

of eukaryotic organisms where a single, nuclear gene encodes the biotin

20   carboxylase, biotin carboxyl carrier protein, and carboxyl transferase domains

within a single, multifunctional polypeptide of 210-280 kDa that, in plants,

forms a dimeric active enzyme. Plant lipid synthesis begins in plastids, where

ACCase synthesizes the malonyl-CoA needed to initiate and elongate fatty acids

of up to 18 carbons (Guenther, 1996). To date, multifunctional ACCase genes

25   from maize, wheat and *Brassica* have been shown to encode functional

chloroplast transit peptides. Both monocots and dicots also appear to contain a

cytoplasmic form of the multifunctional ACCase. The cytoplasmic ACCase may

be involved in the synthesis of malonyl-CoA needed for such reactions as the

synthesis of very long chain fatty acids and flavonoids in plants.

30   The second, multisubunit type of ACCase consists of three

functional domains formed from four separable polypeptides and is found in

dicot plastids and in prokaryotic organisms, but appears not to be present in the

Gramineae. The biotinylated polypeptide component of this multisubunit form

· 96

of ACCase is absent from the *Gramineae* and the plastid-localized

multifunctional ACCase is believed to be responsible for fatty acid biosynthesis.

Measurements of the levels of acetyl-ACP and malonyl-ACP in

spinach leaves (Post-Beittenmiller et al., 1991) and in isolated spinach and pea

5   chloroplasts (Post-Beittenmiller et al, 1991) in the light and dark have provided

evidence that ACCase plays a regulatory role in chloroplast fatty acid

biosynthesis in vegetative tissues. In monocots, there also is evidence that

ACCase activity regulates fatty acid biosynthesis. Several related

cyclohexanedione and aryloxyphenoxypropionate graminicides specifically

10   inhibit monocot multifunctional, plastid-localized ACCase. Complete herbicide

inhibition of ACCase results in plant cell death due to cessation of fatty acid

biosynthesis (Burton et al., 1989). Based on the apparent flux control coefficient

for ACCase in barley and maize leaves exposed to low levels of graminicides,

ACCase was shown to be the major flux-controlling enzyme for light-stimulated

15   lipid synthesis in these leaves (Page et al., 1994). Parker et al. (1990b) isolated

maize tissue cultures that were selected for growth in the presence of normally

lethal concentrations of ACCase inhibiting herbicides. Incubation of wild-type

cell cultures in the presence of herbicide inhibited incorporation of acetate into

the lipid fraction, while variant cell lines exhibiting up to 20-fold increases in

20   wild-type ACCase activity synthesized fatty acid in the presence of herbicide

(Parker et al., 1990b). These results provide further evidence that ACCase is

rate-limiting in monocot vegetative tissues.

Monocot plastid-localized, multifunctional ACCase is likely

involved in regulating seed oil deposition. Herbicide-resistant maize mutant

25   plants were isolated using tissue culture selection (Parker et al., 1990a). The

herbicide-resistant phenotype of these plants is conferred by mutations

converting plastid-localized, multifunctional, herbicide-sensitive ACCase to

herbicide-resistant ACCase (Marshall et al, 1992; Parker et al., 1990a). Two

mutant genes for herbicide-insensitive ACCase, *Acc1* and *Acc2*, have been

30   characterized and both are partially dominant (VanDee et al, 1992). Mutant

alleles of *Acc1* were used to study the role of ACCase in regulating kernel

development and fatty acid synthesis. ACCase activity in corn embryos

increased during development paralleling the reported time course of oil

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deposition in this tissue (Somers et al., 1993). Furthermore, the maximum value of corn embryo ACCase was two-fold greater than the average ACCase activity determined in seedling leaf extracts, suggesting that the enzyme was involved in elevated synthesis of fatty acids required for oil deposition in the embryo. Using

5   the herbicide-insensitivity of the mutant ACCase as a marker for the *Acc1* gene, we determined the tissue-specific expression pattern of that gene. ACCase activity assayed in extracts of developing kernels harvested from herbicide-resistant plants was tested for herbicide inhibition. The embryo and endosperm ACCase activities exhibited similar reductions of herbicide inhibition as ACCase

10   extracted from the leaves of these herbicide-resistant mutants, indicating that the *Acc1* gene was the major ACCase expressed in the embryo and endosperm of the developing corn kernel. To further investigate this gene-enzyme relationship, homozygous herbicide-resistant and wild-type susceptible kernels were isolated four days after pollination and cultured *in vitro* to maturity in the presence and

15   absence of a range of herbicide concentrations (Somers et al., 1993). Wild-type kernels cultured on all levels of herbicide tested were shrunken and ceased development presumably because of herbicide inhibition of kernel ACCase activity and thus fatty acid synthesis. Mutant kernels developed normally in the presence of herbicide, indicating that the *Acc1*-encoded ACCase is the major

20   isoform expressed in the developing kernel. The results from the corn studies indicate that multifunctional, plastic-localized ACCase is a rate-limiting enzyme in fatty acid biosynthesis in vegetative and seed tissues.

Complete coding sequences of several multifunctional ACCases from higher plants have been described. They show high amino acid homology

25   within the ATP, carboxybiotin, and acetyl-CoA binding domains and an overall amino acid similarity of 70% or greater. Maize multifunctional ACCase isoforms are encoded by at least four genes, A1, A2, B1, and B2 (Egli et al., 1997). Type A cDNAs are > 98% identical, and are 95% identical to a type B cDNA. Types A and B encode highly similar N-termini and are both likely to

30   encode plastid-targeted isoforms (Egli and Gengenbach, 1996; Egli et al., 1997; Egli et al., 1995). Herbicide resistance in corn (Marshall et al., 1992; VanDee et al., 1992) has been mapped to the same chromosomal locations as an ACCase cDNA probe with high identity to both A and B types (Caffrey et al., 1995; Egli

et al., 1994; VanDee et al., 1992). Herbicide-tolerant ACCase is expressed in developing corn kernels (Somers et al., 1993) and both A- and B-type cDNAs are found in immature embryos at the time of peak ACCase activity (Egli and Gengenbach, 1996; Somers et al., 1993). In hexaploid wheat, six cDNAs

5 encoding cytosolic ACCase were attributed either to three homoeologous sets of duplicate genes or to allelic differences in each genome (Podkowlinski et al., 1996). Hexaploid wheat also contains three transcriptionally active genes encoding plastidic ACCase with ≥ 98% exon sequence identity. These genes are located on homoeologous chromosomes (Gornicki et al., 1997).

10 Oat is used for both human food and animal feed. Crop improvement goals for oat involve two divergent end uses in that high oil oat is desired as an animal feed whereas a lower oil oat is desired to make low-fat oat products for human consumption. Although progress in decreasing oil content in oat can be achieved by plant breeding, a genetic engineering approach may also

15 be used to create a single, dominant low-oil gene that can be introduced into any oat variety either directly by genetic engineering or via plant breeding for oil reduction in contemporary and future cultivars. Low oil in oat may be a value-added trait for the crop that may allow processors to produce low-fat oat products that contain previously identified cholesterol-lowering constituents

20 including, but not limited to, β-glucans, thus making oats a more healthful human food.

Targeted antisense down-regulation of ACCase in the endosperm using an endosperm-specific promoter may avoid problems that would be caused by inhibition of ACCase in the embryo. Determination of the antisense

25 phenotype by detection of reduced ACCase activity and by quantitation of groat oil content in transgenic lines provides direct evidence for the involvement of ACCase in oat seed oil deposition.

Current oat varieties contain about 7% oil which causes rancidity problems during processing and results in high-fat food products. To reduce oil

30 (triglyceride) content in groats, ACCase cDNA, e.g., oat ACCase DNA, is employed in an antisense orientation to specifically down-regulate oat endosperm ACCase activity and thus reduce groat oil content. Preferably, a plant having a dominantly inherited low-oil gene useful for production of low-oil

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varieties is prepared. As oat oil is primarily localized in the starchy endosperm,

down-regulation of endosperm ACCase activity is less likely to cause deleterious

effects on the embryo. Moreover, converting oil reduction to a single,

dominantly inherited antisense trait is useful in rapid production of low oil

5   varieties in this allohexaploid. Seed from transgenic plants are analyzed for

expression of the transgene during development and for oil content at maturity.

In addition, low oil oat varieties are produced.

            Reduction of groat oil content may decrease processing problems

associated with oil rancidity and can decrease caloric value resulting in a lower

10   fat food. Reduced-fat oatmeal and other oat products may increase the use of oat

as a human food and create a value-enhanced crop for producers of the low-fat

oat. In addition, the preparation of high-energy (high oil) oat feed through use of

a high-oil gene is also contemplated.

Isolation and Characterization of Oat ACCase cDNAs.

15   An oat groat cDNA clone was isolated based on its hybridization

to a maize cDNA fragment which included portions of the less conserved mid-

peptide sequence and the transcarboxylase domain (Shorrosh et al., 1994).

Sequence identity indicated that the oat cDNA (3031 bp) corresponded to maize

ACCase nt 3124-6169 (Egli et al., 1995). The 1010-aa predicted polypeptide

20   was nearly identical to portions of wheat (89%) and maize (84%) plastidic

multifunctional ACCases; it had lower identity to cytosolic ACCases, including

wheat (67.5%) and alfalfa (64.6%) (Gornicki et al., 1994; Shorrosh et al., 1994).

Therefore, the oat-91 cDNA is likely to encode a portion of a plastid-targeted,

multifunctional ACCase polypeptide.

25   An amplified 'Prairie' oat developing groat cDNA library (R.

Skadsen, USDA, Madison, Wisconsin) cloned into the *EcoR*I site of IZAPII

(Stratagene) was screened with a [^{32}P-dCTP]-labeled maize ACCase cDNA

probe (Ausabel et al., 1997) derived from an *EcoR*I digest of clone 18-5 (nt

2020-5927; Egli et al., 1995). One to three plaques/plate of eleven partially

30   purified positives were eluted in SM medium plus CHC13. *In vivo* excision was

carried out to transform SOLR cells with pBlueScript plasmids carrying oat

cDNA inserts, according to the manufacturer's instructions (Stratagene Corp.).

Five colonies per transformation reaction were rescreened (as colony lifts) with

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an AflIII-BbrPI fragment of maize ACCase cDNA (4281-5797; Egli et al., 1995).
One clone, oat-91, hybridized to the probe. Oat-91 was sequenced using the ABI
Prizm system (Perkin-Elmer Corp.) at the Advanced Genetic Analysis Center
(University of Minnesota, St. Paul, Minnesota). Sequence identity indicated the

5   3.05-kb *EcoRI* insert of oat-91 corresponded to maize ACCase nt 3124-6169
(Egli et al., 1995). Oat ACCase cDNA sequence was compared to other plant
ACCases with the BESTFIT program (BESTFIT, Wisconsin Package Version
9.1, Genetics Computer Group (GCG), Madison, Wisconsin).

        For PCR, Pileup alignments of maize, wheat, barley, and rice

10   ACCase coding sequences (GCG, Madison, Wisconsin; 12, 18, F, R) were used
to identify conserved regions (i) for design of new primers or (ii) regions of
identity to available maize ACCase oligos, to be used for PCR amplification of
oat ACCase. Two maize primers, PCR2 (5'-ggcagagcaa agcttggagg) and PCR1
(5'-tccaccagag aagcctctcc) appeared likely to amplify oat cDNA based on their

15   identity to other monocots and previous use to amplify soybean ACCase. These
primers are located within the transcarboxylase domain, surrounding the acetyl-
CoA binding site of ACCase (50). An amplified 'Prairie' oat seeding leaf cDNA
library cloned into the *EcoRI* site of lZAPII (Skadsen, University of Wisconsin)
was used as a source of cDNA. One I of the oat cDNA library was amplified

20   with maize primers (PCR2/PCR1), using the Expand High Fidelity PCR system
(BMB) in a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer) after
denaturing phage heads at 94°C for 10 minutes. The single, 222-bp product was
purified by Wizard PCR Plus (Promega Corp.) and sequenced in an AB1377
(Perkin-Elmer) using PCR1 as primer. The resulting sequence appeared to be

25   from oat since it had high identity to maize plastidic ACCase (89%; 4, 12, Egli,
unpublished). Additional 5' cDNA was obtained by (i) PCR of the oat cDNA
library with T3/PCR1 primers to generate a complex band mixture and (ii)
reamplification of 0.1 I products with maize ACCase primers 1324+ (5'-
caatcacgtcagactg)/PCR1 to obtain a 1.9-kb product with the same 3' sequence as

30   the PCR2/PCR1 product.

        To test whether the oat ACCase cDNA clone oat-91 encodes a
plastidic isoform, 422 nt of 3' insert sequence was translated and compared to
predicted amino acid sequences of maize plastidic ACCases, a putative maize

cytosolic ACCase, and to multifunctional ACCases from wheat, barley, alfalfa, soybean, *Arabidopsis*, *Brassica*, chicken, human, *Ustilago*, yeast and *Cyclotella*. Aligned, degapped peptides were used to construct a phylogram by the UPGMA method based on Kimura protein distances (GCG, Madison, Wisconsin). The

5   oat ACCase sequence was ≥ 93% identical to plastidic maize and wheat ACCase sequences, but only 78-83% identical to cytosolic ACCases from maize, wheat, or dicots. The corresponding nucleotide identities were 88-92% and 73-78%, respectively. In the phylogram, monocot ACCases were separated from dicots and grouped according to their known or expected cellular locations (Figure 25).

10   Both sequence identity and phylogenetic grouping indicate that oat cDNA encodes a plastidic ACCase.

Additional 5' sequence is obtained by screening the cDNA library with the most 5' oat probes or by PCR of the library with near 5' (upstream) oat plus vector-specific oligonucleotide primers. Alternatively, the 5' end of

15   ACCase message may be obtained by 5' RACE PCR (Frohman, 1993). Total RNA is isolated from developing groats, and digested with DNAse. Complementary DNA is synthesized with Superscript II reverse transcriptase (Gibco BRL) by priming with oat ACCase primers. PCR amplification of cDNA utilizes oat primers plus 5' maize primers. Because chloroplast transit peptide

20   sequences are not well conserved between ACCase genes of maize, wheat, and *Brassica*, or between different gene families of a single species, and because 5' non-coding sequences also are unlikely to be conserved, completion of the 5' sequence of oat ACCase cDNA is accomplished by 5' RACE-PCR (GIBCO-BRL; Gornicki et al., 1994). 5' RACE products and downstream sequences are

25   sequenced to verify that a chloroplast transit peptide coding sequence is present and to identify primers that may be used to generate a complete or partial oat ACCase cDNA product by PCR. Oat cDNA is reamplified using either the Expand High Fidelity or Long Template PCR kits (Boehringer Mannheim Biochemicals), depending on the length of desired product. Products are blunt-

30   cloned into pBluescript (Stratagene) or a similar vector, or extended with dATP and cloned into pGEM-T (Promega). Adjacent sites in vector polycloning regions or unique sites introduced by PCR are utilized to move oat ACCase cDNA into expression vectors.

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Co-segregation of Oat ACCase cDNA and a Major Groat Oil QTL

 A major quantitative trait locus (QTL) controlling groat oil

content in oat was recently identified by the oat genome mapping program at the

University of Minnesota (See Example XIV). Single factor ANOVA showed

5 that RFLP marker *Xcdo665B* on linkage group 11 was associated with a QTL

accounting for approximately 37% of the variance in groat oil in a Kanota by

Ogle mapping population (O'Donoughue et al., 1995). The 1.9 kb oat ACCase

cDNA was placed on this map to within 2.6 cM of *Xcdo665B* in the same

population (n = 136). This ACCase polymorphism also accounted for about

10 37% of the variation in groat oil content. These results provide strong support

for the hypothesis that ACCase has a major role in determining groat oil content.

Either allelic variation in ACCase activity controls oil content in oat groats or a

gene(s) closely linked to ACCase is responsible. Suppression of ACCase

expression by antisense transformation can provide a direct test of whether

15 ACCase activity indeed has a major effect on groat oil.

Antisense ACCase Transformation Construct Production

 Antisense ACCase-cDNAs are constructed using standard

procedures in the plasmids that are routinely used for oat transformation (Torbert

et al., 1995). The cDNA is ligated in antisense orientation relative to an

20 endosperm-specific promoter and is terminated using the nopaline synthase

(NOS) 3' untranslated sequence of the *Agrobacterium tumefaciens* NOS gene,

although other termination regions may be employed. This antisense cassette is

ligated into a plasmid backbone carrying a neomycin phosphotransferase II

(nptII) gene under control of the cauliflower mosaic virus 35S promoter as a

25 selectable marker (Torbert et al., 1995).

 An investigation of down-regulated phenotypes conferred by

expression of various granule-bound starch synthase antisense constructs in

potato indicated that the size of the antisense transcript did not affect efficacy of

inhibition (Kuipers et al., 1995). Choice of promoter sequences controlling

30 expression of the antisense construct appeared to have important effects in the

degree of antisense inhibition of potato granule-bound starch synthase. Effective

antisense RNAs have been reported which (relative to the mRNA) are either full

length or fragments designed to block 5' end capping, intron splicing, or the poly

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A tail (Guerineau et al., 1994; Inoye, 1988). Preferably, the longest oat ACCase clones are employed in an anti-sense construct.

Complete inhibition of vegetative- or seed-expressed ACCase is likely to be lethal because the enzyme is required for general fatty acid

5   biosynthesis, as shown by the lethality of herbicide inhibition of ACCase in susceptible monocots. In oat, it is not known whether tissue-specific isozymes or homologs of ACCase exist, or if they do, whether they vary significantly in nucleotide sequence. Therefore, it is important to target antisense down-regulation of ACCase to the developing oat endosperm and that the antisense

10   gene exert a partial down-regulation of endosperm ACCase homologs to avoid deleterious effects of possible endosperm ablation due to complete inhibition of ACCase. Because antisense suppression of heterologous genes or members of a gene family can occur (deLang et al., 1993), and because the partial inhibition of plastidic ACCases but not cytosolic ACCases is desired, partial antisense cDNAs

15   designed from functional domain sequences may be used, or conversely, less-conserved regions between functional domains. Comparison of wheat genes for cytosolic and plastidic ACCases (Gornicki et al., 1997; Gornicki et al., 1994) may aid in the design of antisense cDNAs to specifically target plastidic ACCase.

20   The wheat Dy10 high molecular weight glutenin promoter (Belchi and Anderson, 1994) confers strong expression of GUS in oat and expression is specifically limited to the endosperm. Because of its tissue specificity and the likelihood that its expression pattern does not directly coincide with the expression of ACCase during oat endosperm development, the Dy10 promoter

25   may be useful in restricting the ACCase antisense transcripts to the oat endosperm resulting in a partial inhibition of ACCase expression and the normal development of endosperm cells.

Oat Transformation

Elite cultivars of oat can be transformed and on average one

30   transgenic tissue culture is produced that regenerates fertile, transgenic plants per two microprojectile bombardments. Generally, from 15 to 30 transgenic plants are produced from the microprojectile bombardments conducted during one experiment. The oat ACCase antisense plasmid is introduced into regenerable

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oat tissue cultures of oat genotypes that exhibit high oil content using

microprojectile bombardment as described by Torbert et al. (1995). Tissue

cultures are selected for resistance to paromomycin conferred by the nptII

selectable marker gene. Paromomycin-resistant tissue cultures are regenerated

5   and plants analyzed for the nptII protein using an NPTII ELISA kit (3' > 5' Inc.,

Boulder, CO). Plants that express the nptII protein are grown to maturity.

The progeny from at least 100 independent transgenic events are

then evaluated.

Characterization of the ACCase Antisense Phenotype

10   Homozygous T_2 seed are divided for maintenance, advancement

to produce greater quantities of seed for more detailed analyses, and for

preliminary analysis. Single oat plants may produce up to 100 seed. Therefore,

sufficient seed should be available to determine if a transgene phenotype is

detectable. Dried mature seed of antisense homozygotes and nontransgenic

15   sister lines are analyzed for oil content initially using nondestructive Near

Infrared Reflectance (NIR) spectroscopy. This method requires only 1 g of

dehulled groats and is inexpensive. NIR is employed to rapidly identify

transgenic lines that exhibit lowered oil content. Selected lines identified by

NIR are reanalyzed using either NMR or the gravimetric procedure (Peterson

20   and Wood, 1997). If differences in oil content are observed, oil composition is

analyzed by gas chromatography. If the results of these analyses warrant further

investigation of the materials, a replicated greenhouse trial is conducted to

confirm the reduced oil phenotype in the transgenic plants. This test is

conducted on T_3 seed produced from multiple T_2 plants of each transgenic line.

25   If a low-oil phenotype is observed, these materials are entered into a breeding

program to initiate variety development.

Selected low-oil lines are advanced an additional generation.

Seed is isolated at different stages of development and stored under conditions to

preserve ACCase activity and mRNA for Northern blot analysis. ACCase

30   activity levels and sense and antisense transcript levels are determined to further

characterize the antisense phenotype of these plants.

While oat is considered a beneficial alternative crop with multiple

uses, production acreage continues to shrink due to poor profitability and

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international competition. Yet oat is used as a high quality animal feed and for

production of nutritious human food. Numerous positive human health qualities

are attributed to consumption of oatmeal. Foremost among these is the

cholesterol-lowering property of oats. A major problem confronting oat

5   processors is that current oat varieties have elevated oil content that precludes

the production of a low-fat oat product. Thus, in the production of oat products

such as oatmeal, the high oil content offsets the benefit of the cholesterol-

lowering constituents of the product. Oat oil content is relatively easy to

manipulate using conventional plant breeding. However, the trend has been to

10   increase oil content of oats for use as animal feed and only recently has low-oil

oat been recognized as important for food products.

Thus, oat varieties with reduced oil content may be prepared

using genetic engineering. Moreover, a single dominant gene system can vastly

simplify conversion of existing agronomically adapted oat varieties to low oil as

15   well as being useful in the production of new low-oil varieties.

Based on the demand by processors for low-oil oat varieties, it is

warranted to consider low oil as a value-added trait for oat. Increasing the value

of oat may increase the acreage devoted to growing oats in the U.S. because of

possible increased profitability of the crop.

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References

Abu-Elheiga, L., Jayakumar, A., Baldini, A., Chirala S. S., Wakil, S. J.

Human acetyl-CoA carboxylase: characterization, molecular cloning and

evidence for two isoforms. Proc. Nat. Acad. Sci. U.S.A., 92:4011-4015 (1995).

25   Ahn, S., Tanksley, S. D. Comparative linkage maps of the rice and

maize genomes. Proc. Natl. Acad. Sci. U.S.A., 90:7980-7984 (1993).

Alban, C., Baldet, P., Douce, R. Localization and characterization of two

structurally different forms of acetyl-CoA carboxylase in young pea leaves, of

which one is   sensitive to aryloxyphenoxypropionate herbicides. Biochem. J.,

30   300:557-565 (1994).

106

Al-Feel, W., Chirala, S. S., Wakil, S. J. Cloning of the yeast FAS3 gene and primary structure of yeast acetyl-CoA carboxylase. Proc. Natl. Acad. Sci. U.S.A., 89:4534-4538 (1992).

Alrefai, R., Berke, T. G., Rocheford, T. R. Quantitative trait locus analysis of fatty acid concentrations in maize. Genome, 38:894-901 (1995).

Anderson, J. V., Lutz, S. M., Gengenbach, B. G., Gronwald, J. W. Genomic sequence for a nuclear gene encoding acetyl-coenzyme A carboxylase (GenBank L42814) in soybean. Plant Physiol., 109:338 (1995).

Ashton, A., Jenkins, C. L. D., Whitfeld, P. R. Molecular cloning of two different cDNAs for maize acetyl-CoA carboxylase. Plant Mol. Biol., 24:35-49 (1994).

Ausubel F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., eds. Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1997).

Bailey, A., Keon, J., Owen, J., Hargreaves, J. The ACC1 gene, encoding acetyl-CoA carboxylase, is essential for growth in Ustilago maydis. Mol. Gen. Genet., 249:191-201 (1995).

Belchl, A. E., Anderson, O. D. Expression of a novel high-molecular-weight glutenin subunit gene in transgenic wheat. Nature Biotechnology, 14 (1996).

Berke, T.G., Rocheford, T.R. Quantitative trait loci for flowering, plant and ear height, and kernel traits in maize, Crop Sci., 35, 1542-1549 (1995).

Betty, M., Ireland, R. J., Smith, A. M. Purification and characterization of acetyl-CoA carboxylase from developing pea embryos. Plant Physiol. J., 140:513-520 (1992).

Branson, V., Frey, K. J. Recurrent selection for groat oil content in oat. Crop Sci., 28:1382-1387 (1989).

Brummer, E. C., Graef, G. L., Orf, J., Wilcox, J. R., Shoemaker, R. C. Mapping QTL for seed protein and oil content in eight soybean populations. Crop Sci., 37:370-378 (1997).

Burton, J. D., Gronwald, J. W, Somers, D. A., Gengenbach, B. G., Wyse, D. L. Inhibition of corn acetyl-CoA carboxylase by cyclohexanedione and aryloxyphenoxypropionate herbicides. Pest Biochem. Physiol., 34:76-85 (1989).

Bush, A. L., Wise, R. P., Rayapati, P. J., Lee, M. Restriction fragment length polymorphisms linked to genes for resistance to crown rust (*Puccinia coronata*) in near-isogenic lines of hexaploid oat (*Avena sativa*). Genome, 37:823-831 (1994).

Caffrey, J. J., Wurtele, E. S., Nikolau, B. J. Genetic mapping of two acetyl-CoA carboxylase genes. Maize Genetics Cooperation Newsletter, 69:3-4 (1995).

Cakir, M., Reeves, D. L., Kahler, A. L. Linkage analysis of RFLP markers and quantitative traits in hexaploid oats. Agronomy Abstracts. American Society of Agronomy, Madison, Wisconsin, pp. 163 (1996).

Charles, D. J., Cherry, J. H. Purification and characterization of acetyl-CoA carboxylase from developing soybean seeds. Phytochem., 25:1067 (1986).

de Lange, P., de Boer, G-J, Mol, J. M. N., Kooter, J. M. Conditional inhibition of β-glucuronidase expression by antisense gene fragments in petunia protoplasts. Plant Molec. Biol., 23:45-55 (1993).

Diers, B. W., Keim, P., Fehr, W. R., Shoemaker, R. C. RFLP analysis of soybean seed protein and oil content. Theor. Appl. Genet., 83:608-612 (1992).

Dudley, J. W., Lambert, R. J., Alexander, D. E. Seventy generations of selection for oil and protein concentration in the maize kernel. In: Dudley, J. W. (ed.) Seventy generations of selection for oil and protein concentration in the maize kernel. Crop Sci. Soc. Am., Madison, Wisconsin, pp. 181-212 (1974).

Egli, M., Gengenbach, B. Plastid localization of a multifunctional acetyl-CoA carboxylase in maize. Maize Genetics Cooperation Newsletter, 70:58-59 (1996).

Egli, M. A., Gengenbach, B. G., Gronwald, J. W., Somers, D. A., Wyse, D. L. Characterization of maize acetyl-coenzyme A carboxylase. Plant Physiol., 101:499-506 (1993).

Egli, M., Lutz, S., Gengenbach, B. Genetic analysis of maize plastidic acetyl-CoA carboxylase. 1997 Biochemistry and Molecular Biology of Plant Fatty Acid and Glycerolipids Symposium, South Lake Taho, CA, P-4, (1997).

Egli, M., Lutz, S., Somers, D., Gengenbach, B. Identification and mapping of maize acetyl-CoA carboxylase genes. Maize Genetics Cooperation Newsletter, 68:92-93 (1994).

Egli, M. A., Lutz, S. M., Somers, D. A., Gengenbach, B. G. A maize acetyl-coenzyme A carboxylase cDNA sequence. Plant Physiol., 108:1299-1300 (1995).

Elborough, K. M., Simon, J. W., Swinhoe, R., Ashton, A. R., Slabas, A. R. Studies on wheat acetyl CoA carboxylase and the cloning of a partial cDNA. Plant Mol. Biol.,24:21-34 (1994).

Evenson, K. J., Gronwald, J. W., Wyse, D. L. Purification and characterization of acetyl-coenzyme A carboxylase from diclofop-resistant and -susceptible *Lolium multiflorum*. Plant Physiol., 105:671-680 (1994).

Frohman, M. A. Rapid amplification of complementary DNA ends for generation of full-length complementary DNAs: Thermal RACE. Methods in Enzymology, 218:340-356 (1993).

Gale, M. D., Devos, K. M., Proc. Natl. Acad. Sci. USA, 95, 1971 (1998).

Goldman, I. L., Rocheford, T. R., Dudley, J. W. Quantitative trait loci influencing protein and starch concentration in the Illinois Long Term Selection maize strains. Theor. Appl. Genet., 87:217-224 (1993).

Goldman, I. L., Rocheford, T. R., Dudley, J. W. Molecular markers associated with maize kernel oil concentration in an Illinois high protein X Illinois low protein cross. Crop. Sci., 34:908-915 (1994).

Gornicki, P., Faris, J., King, I., Podkowinski, J., Gill, B., Haselkorn, R. Plastid-localized acetyl-CoA carboxylase of bread wheat is encoded by a single gene on each of the three ancestral chromosome sets. Proc. Natl. Acad. Sci. U.S.A., 94:14179-14184 (1997).

Gornicki, P., Haselkorn, R. Wheat acetyl-CoA carboxylase. Plant Mol. Biol.,22:547-552 (1993).

Gornicki, P., Podkowinski, J., Scappino, L. A., DiMaio, J., Ward, E., Haselkorn, R. Wheat acetyl-coenzyme A carboxylase: cDNA and protein structure. Proc. Natl. Acad.Sci. U.S.A., 91:6860-6864 (1994).

Gornicki, P., Faris, J., King, I., Podkowinski, J., Gill, B., Haselkorn, R. Plastid-localized acetyl-CoA carboxylase of bread wheat is encoded by a single gene on each of the three ancestral chromosome sets, Proc. Nat. Acad. Sci. USA, 94, 14179-14184 (1997).

109

Gornicki, P., Podkowinski, J., Scappino, L.A., DiMaio, J., Ward, E., Haselkorn, R. Wheat acetyl-coenzyme A carboxylase: cDNA and protein structure, Proc. Natl. Acad. Sci. USA, 91, 6860-6864 (1994).

Guenther, S. Genbank accession #X99102 (barley) (1996).

5   Guerineau, F., Waugh, R. Brown, J. W. S. Parameters affecting the activity of antisense RNA sequences in tobacco protoplasts. Plant Cell Rep., 13:703-708 (1994).

Guchhait, R. B., Polakis, E., Dimroth, P., Stoll, E., Moss, J., Lane, M. D. Acetyl coenzyme A carboxylase system of Escherichia coli. J. Biol. Chem.,
10   249:6633-6645 (1974).

Gullord, M. Oil and protein content in oats (Avena sativa L.). In: Lawes, D. A., and Thomas, H. (eds.) Proc. 2nd Int. Oats Conf. Aberystwyth, Wales. 15-18 July 1985. Martinus Nijhoff Publishers, Dordrecht, The Netherlands, pp. 210-213 (1986).

15   Harwood, J. L. Fatty acid metabolism. Ann. Rev. Plant Physiol. Plant Mol. Biol., 39:101-138 (1988).

Holland, J. B., Moser, H.S., O'Donoughue, L. S., Lee, M. QTLs and epistasis associated with vernalization responses in oat. Crop Sci., 37:1306-1316 (1997).

20   Hosaka, K., Kianian, S. F., McGrath, J. M., Quiros, C. F. Development and chromosomal localization of genome-specific DNA markers of Brassica and the evolution of amphidiploids and n = 9 diploid species. Genome, 33:131-142 (1990).

Hu, J., Quiros, C. F., Arus, P., Struss, D., Robbelen, G. Mapping of a
25   gene determining linolenic acid concentration in rapeseed with DNA-based markers. Theor. Appl. Genet., 90:258-262 (1995).

Inouye, M. Antisense RNA: its functions and applications in gene regulation – a review. Gene, 72:25-34 (1988).

Keegstra, K., Olsen, L. J., Theg, S. M. Chloroplastic precursors and their
30   transport across the envelope membranes. Annu. Rev. Plant Physiol. Plant Mol. Biol., 40:471-501 (1989).

Kennard, W. C., Havey, M. J. Quantitative trait analysis of fruit quality in cucumber: QTL detection, confirmation, and comparison with mating-design variation. Theor. Appl. Genet., 91:53-61 (1995).

Kennard, W.C., Slocum, M.K., Figdore, S.S., Osborn, T.C. Genetic analysis of morphological variation in *Brassica oleracea* using molecular markers, Theor. Appl. Genet., 87, 721-732 (1994).

Kianian, S. F., Wu, B-C, Fox, S. L., Rines, H. W., Phillips, R. L. Aneuploid marker assignment in hexaploid oat with the C genome as a reference for determining remnant homoeology. Genome, 40:386-396 (1997).

Kolattukudy, P. E. Chemistry and Biochemistry of Natural Waxes. P. E. Kolattukudy, ed., Elsevier, New York, pp. 289-347 (1976).

Kondo, H., Shiratsuchi, K., Yoshimoto, T., Mauda, T., Kitazono, A., Tsuru, D., Anai, M., Sekiguchi, M., Tanabe, T. Acetyl-CoA carboxylase from *E. coli*: gene organization and nucleotide sequence of the biotin carboxylase subunit. Proc. Natl. Acad. Sci. U.S.A., 88:9730-9733 (1991).

Konishi, T., Shinohara, K., Yamada, K., Sasaki, Y. Acetyl-CoA carboxylase in higher plants: most plants other than Gramineae have both the prokaryotic and the eukaryotic forms of this enzyme. Plant Cell Physiol., 37:117-122 (1996).

Kuipers, A. G. S., Soppe, W. J. J., Jacobsen, E., Visser, R. G. F. Factors affecting inhibition by antisense RNA of granule-bound starch synthase gene expression in potato. Mol. Gen. Genet., 246:745-755 (1995).

Lee, E-S J., Gronwald, J. W., Orf, J. H. Acetyl-CoA carboxylase activity in high-oil and low-oil soybeans (*Glycine max* L.). 11th International Meeting on Plant Lipids. Paris, France. Abstract A14 (1994).

Li, S-J, Cronan, J. E. The gene encoding the biotin carboxylase subunit of *E. coli* acetyl-CoA carboxylase. J. Biol. Chem., 267:855-863 (1992).

Li, S-J, Cronan, J. E. The genes encoding the two carboxyltransferase subunits of *E. coli* acetyl-CoA carboxylase. J. Biol. Chem., 267:16841-16847 (1992).

Lopez-Casillas, F., Bai, D-H, Luo, X., Kong, I-S, Hermodson, M. A., Kim, K-H. Structure of the coding sequence and primary amino acid sequence

111

of acetyl-coenzyme A carboxylase. Proc. Natl. Acad. Sci. U.S.A., 85:5784-5788 (1988).

Mansur, L. M., Lark, K. G., Kross, H., Oliveira, A. Interval mapping of quantitative trait loci for reproductive, morphological, and seed traits of soybean (*Glycine max* L.). Theor. Appl. Genet., 86:907-913 (1993).

Marshall, L. C., Somers, D. A., Dotray, P. A., Gengenbach, B. G., Wyse, D. L., Gronwald, J. W. Allelic mutations in acetyl-coenzyme A carboxylase confer herbicide tolerance in maize. Theor. Appl. Genet., 83:435-442 (1992).

Marten, G. C., Shenk, J. S., Barton II, F. E. (ed.) Near infrared reflectance spectroscopy (NIRS): Analysis of forage quality. Agric. Handb. 643. USDA-ARS, Washinton, D. C. (1989).

Morrison, W. R. Cereal lipids. In: Pomeranz, Y. (ed.) Advances in cereal science and technology. Vol. 2. Am. Assoc. Cereal Chem., St. Paul, Minnesota, pp. 221-348 (1978).

Nikolau, B.J., Hawke, J.C. Purification and characterization of maize leaf acetyl-CoA carboxylase, Arch. Biochem. Biophys., 228, 86-96 (1984).

O'Donoughue, L. S., Kianian, S. F., Rayapati, P. J., Penner, G. A., Sorrells, M. E., Tanksley, S. D., Phillips, R. L., Rines, H. W., Lee, M., Fedak, G., Molnar, S. J., Hoffman, D., Salas, C. A., Wu, B., Autrique, E., Van Deynze, A. A molecular linkage map of cultivated oat. Genome, 38:368-380 (1995).

Ohlrogge, H. B., Jaworski, J. B., Regulation of fatty acid synthesis, Annu. Rev. Plant Physiol. Plant Mol. Biol., 48, 109-136 (1997).

Page, R. A., Okada, S., Harwood, J. L. Acetyl-CoA carboxylase exerts strong flux control over lipid synthesis in plants. Biochem. Biophys. Acta, 1210:369-372 (1994).

Page, R.A., Okada, S., Harwood, J.L. Acetyl-CoA carboxylase exerts strong flux control over lipid synthesis in plants, Biochem. Biophys. Acta, 1210, 369-372 (1994).

Parker, W. B., Marshall, L. C., Burton, J. B., Somers, D. A., Wyse, D. L., Gronwald, J. W., Gengenbach, B. G. Dominant mutations causing alterations in acetyl-coenzyme A carboxylase confer tolerance to cyclohexanedione and aryloxyphenoxypropionate herbicides in maize. Proc. Natl. Acad. Sci. U.S.A., 87:7175-7179 (1990a).

Parker, W. B., Somers, D. A. Wyse, D. L., Keith, R. A., Burton, J. D., Gronwald, J. W., Gengenbach, B. G. Selection and characterization of sethoxydim-tolerant maize tissue cultures. Plant Physiol., 92:1220-1225 (1990b).

5   Peterson, D. M., Wood, D. F. Composition and structure of high oil oat. J. Cereal Sci., 26:121-128 (1997).

Phillips, R. L., Vasil, I. K. (eds.) DNA-based markers in plants. Kluwer Academic Publishers. Dordrecht, The Netherlands (1994).

Podkowinski, J., Sroga, G. E., Haselkorn, R., Gornicki, P. Structure of a

10   gene encoding a cytosolic acetyl-CoA carboxylase of hexaploid wheat. Proc. Natl. Acad. Sci. U.S.A., 93:1870-1874 (1996).

Post-Beittenmiller, D. J., Jaworski, J. G., Ohlrogge, J. B. In vivo pools of free and acylated acyl carrier proteins in spinach. Evidence for sites of regulation of fatty acid biosynthesis. J. Biol. Chem., 226:1858-1865 (1991).

15   Post-Beittenmiller, D., Roughan, P. G., Ohlrogge, J. B. Regulation of plant fatty acid biosynthesis: Analysis of acyl-coenzyme A and acyl-acyl carrier substate pools in spinach and pea chloroplast. Plant Physiol., 100:923-930 (1992).

Rajhathy, T., Thomas, H. Cytogenetics of oats (Avena L.). Wilkes, A.

20   (ed.). Genetics Society of Canada Miscellaneous Publication No. 2. Genetics Society of Canada, Ottawa, Ontario (1974).

Ripsin, C. M., Keenan, J. M., Jacobs, D. R., Elmer, P. J., Welch, R. R., Van Horn, L., Liu, K., Turnbull, W. H., Thye, F. W., Kestin, M., Hegsted, M., Davidson, D. M., Davidson, M. H., Dugan, L. D., Demark-Wahnefried, W.,

25   Beling, S. Oat products and lipid lowering. J. Am. Med. Assoc., 267:3317-3325 (1992).

Robertson, D. S. A possible technique for isolating genic DNA for quantitative traits in plants. J. Theor. Biol., 117:1-10 (1985).

Rocheford, T. R. Chromosome Regions Associated with Control of

30   Maize Kernel Composition. Proceedings of the 49th Annual Sorghum Research Conference, pp. 239-249 (1994).

Roesler, K., Shintani, D., Savage, L., Boddupalli, S., Ohlrogge, J.

Targeting of the *Arabidopsis* homomeric acetyl-coenzyme A carboxylase to

plastids of rapeseeds. Plant Physiol., 113:75-81 (1997).

Roesler, K. R., Shorrosh, B. S., Ohlrogge, J. B. Structure and expression

5   of an *Arabidopsis* acetyl-coenzyme A carboxylase gene. Plant Physiol.,

105:611-617 (1994).

Roessler, P. G., Ohlrogge, J. B. Cloning and characterization of the gene

that encodes acetyl-coenzyme A carboxylase in the alga *Cyclotella cryptica*. J.

Biol. Chem.,268:19254-19259 (1993).

10   Rooney, W. L., Jellen, E. N., Phillips, R. L., Rines, H. W., Kianian, S. F.

Identification of homoeologous chromosomes in hexaploid oat (*A. byzantina* cv.

Kanota) using monosomics and RFLP analysis. Theor. Appl. Genet., 89:329-

335 (1994).

Sambrook, J., Fritsch, E.F., Maniatis, T. Molecular cloning: A laboratory

15   manual, 2nd ed., Cold Spring Harbor Laboratory Press (1989).

Siripoonwiwat, W., O'Donoughue, L.S., Wesenberg, D., Hoffman, D.L.,

Barbosa-Neto, J.F., Sorrells, M.E. Chromosomal regions associated with

quantitative traits in oats. JQTL

(http://probe.nalusda.gov:8000/otherdocs/jqtl/jqtl1996-03) (1996).

20   SAS Institute, Inc. SAS procedures guide, Version 6, 3rd ed. SAS

Institute Inc., Cary, N.C. (1990).

Schipper, H., Frey, K. J. Selection for groat-oil content in oats grown in

field and greenhouse. Crop Sci., 31:661-665 (1991a).

Schipper, H. Frey, K. J. Observed gains for three recurrent selection

25   regimes for increased groat-oil content. Crop Sci., 31:1505-1510 (1991b).

Schrickel, D. J. Oat production, value and use. In: Webster F. H. (ed.)

Oat chemistry and technology. Assoc. Cereal Chem.,St. Paul, Minnesota, pp. 1-

11 (1986).

Schulte, W., Schell, J., Toepfer, R. A gene encoding acetyl-coenzyme A

30   carboxylase from *Brassica napus*. Plant Physiol., 106:793-794 (1994).

Schulte, W., Topfer, R., Stracke, R., Schell, J., Martini, N. Multi-

functional acetyl-CoA carboxylase from *Brassica napus* is encoded by a multi-

gene family: Indication for plastidic localization of at least one isoform. Proc. Natl. Acad. Sci. U.S.A., 94:3465-3470 (1997).

Sears, E. R. The nature of mutation in hexaploid wheat. Symp. Biol. Hung., 12:73-82 (1972).

Shintani, D., Roesler, K., Shorrosh, B., Savage, L., Ohlrogge, J. Antisense expression and overexpression of biotin carboxylase in tobacco leaves. Plant Physiol., 114:881-886 (1997).

Shorrosh, B. S., Dixon, R. A., Ohlorogge, J. B. Molecular cloning, characterization, and elicitation of acetyl-CoA carboxylase from alfalfa. Proc. Natl. Acad. Sci. U.S.A., 91:4323-4327 (1994).

Simcox, P. D., Gerland, W., De Luca, V., Canvin, D. T., Dennis, D. T. Respiratory pathways and fat synthesis in the developing castor oil seed. Can. J. Bot., 57:1008-1014 (1979).

Siripoonwiwat, W., O'Donoughue, L. S., Wesenberg, D., Hoffman, D. L., Barbosa-Neto, J. F., Sorrells, M. E. Chromosomal regions associated with quantitative traits in oat. JQTL (http://probe.nalusda.gov:8000/otherdocs/jqtl/jqtl1996-03) (1996).

Slabas, A. R., Fawcett, T. The biochemistry and molecular biology of plant lipid biosynthesis. Plant Mol. Biol., 19:169-191 (1992).

Slabas, A. R., Hellyer, A., Bambridge, H. E. The basic polypeptide subunit of rape leaf acetyl-CoA carboxylase is a 220 kDa protein. Biochem. Soc. Trans., 14:714-715 (1986).

Somers, D. A., Keith, R. A., Egli, M. A., Marshall, L. C., Gengenbach, B. G., Gronwald, J. W., Wyse, D. L. Expression of the Acc1-gene-encoded acetyl-coenzyme-A carboxylase in developing maize (Zea mays L.) kernels. Plant Physiol., 101:1097-1101 (1993).

Somers, D. A., Rines, H. W., Gu, W., Kaeppler, H. F., Bushnell, W. R. Fertile, transgenic oat plant. Bio/Technology, 10:1589-1594 (1992).

Somers, D. A., Torbert, K. A., Pawlowski, W. P., Rines, H. W., Progress in genetic engineering of oat. Proceedings of the 5th International Oat Conference and the 7th International Barley Genetics Symposium, Scoles, G., Rossnagel, B., eds. University Extension Press, University of Saskatchewan, pp. 230-234 (1996).

Takai, T., Yokayama, C., Wada, K., Tanabe, T. Primary structure of chicken liver acetyl-CoA carboxylase deduced from cDNA sequence. J. Biol. Chem., 263:2651-2657 (1988).

Tanhuanpaa, P. K., Vikki, J. P., Vikki, H. J. Identification of a RAPD marker for palmitic-acid concentration in the seed of spring turnip rape (Brassica rapa spp. oleifera). Theor. Appl. Genet., 91:477-480 (1995a).

Tanhuanpaa, P. K., Vikki, J. P., Vikki, H. J. Association of a RAPD marker with linolenic acid concentration in the seed oil of rapeseed (Brassica napus L.). Genome, 38:414-416 (1995b).

Thro, A. M., Frey, K. J. Inheritance of groat-oil content and high-oil selection in oats (Avena sativa L.). Euphytica, 34:251-263 (1985).

Tinker, N. A., Mather, D. E. Methods for QTL analysis with progeny replicated in multiple environments. JQTL (http://probe.nalusda.gov:8000/otherdocs/jqtl/jqtl1995-01) (1995a).

Tinker, N. A., Mather, D. E. MQTL: software for simplified composite interval mapping of QTL in multiple environments. JQTL (http://probe.nalusda.gov:8000/otherdocs/jqtl/jqtl1995-02) (1995b).

Tinker, N. A., Mather, D. E., Rossnagel, B. G., Kasha, K. J., Kleinhofs, A., Hayes, P. M., Falk, D. E., Ferguson, T., Shugar, L. P., Legge, W. G., Irvine, R. B., Choo, T. M., Briggs, K. G., Ullrich, S. E., Franckowiak, J. D., Blake, T. K., Graf, R. J., Dofing, S. M., Saghai Maroof, M. A., Scoles, G. J., Hoffman, D., Dahleen, L. S., Kilian, A., Chen, F., Biyashev, R. M., Kudrna, D. A., Steffenson, B. J. Regions of the genome that affect agronomic performance in two-row barley. Crop Sci., 36:1053-1062 (1996).

Torbert, K. A., Rines, H. W., Somers, D. A. Use of paramomycin as a selective agent for oat transformation. Plant Cell Reports, 14:635-640 (1995).

Torbert, K. A., Rines, H. W., Somers, D. A. Transformation of oat using mature embryo-derived tissue cultures. Crop Sci., 38:226-231 (1998).

Turnham, E., Northcote, D. N. Changes in the activity of acetyl-CoA carboxylase during rapeseed formation. Biochem. J., 212:223-229 (1983).

Van Dee, K. L., Somers, D. A., Wyse, D. L. RFLP mapping of Acc1 in Zea mays L. Agronomy Abstracts, p.198 (1992).

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Van Dee, K.L. Evidence for two independently segregating loci encoding acetyl-CoA carboxylase in *Zea mays*, M.S. Thesis, Univ. of Minnesota, 62 p. (1994).

VanDeynze, A. E., Dubcovsky, J., Gill, K. S., Nelson, J. C., Sorrells, M.
5   E., Dvorak, J., Gill, B. S., Lagudah, E. S., McCouch, S. R., Appels, R. Molecular-genetic maps for group 1 chromosomes of Triticeae species and their relation to chromosomes in rice and oat. Genome, 38:45-59 (1995).

Webster, F. H. Oat utilization: past, present and future. In: Webster F. H. (ed.) Oat chemistry and technology. Assoc. Cereal Chem., St. Paul,
10   Minnesota, pp. 413-427 (1986).

Welch, R. W. The chemical composition of oats. In: The Oat Crop, Welch, R.W., ed. Chapman and Hall, pp. 279-320 (1995).

Youngs, V. L. Oat lipids and lipid in related enzymes. In: Oats: Chemistry and Technology, Webster, F. H., ed. American Association of Cereal
15   Chemists, Inc. pp. 205-226 (1986).

Youngs, V. L. Oat lipids and lipid-related enzymes. In: Webster, F. H. (ed.) Oat chemistry and technology. Assoc. Cereal Chem., St. Paul, Minnesota, pp. 205-226 (1986).

Youngs, V. L., Peterson, D. M., Brown, C. M. Oats. In: Pomeranz, Y.
20   (ed.) Advances in cereal science and technology. Vol. 5. Am. Assoc. Cereal Chem., St. Paul, Minnesota, pp. 49-105 (1982).

Yuzo, M., Takuji, S. Genbank accession #D24786 (rice) (1993).

The complete disclosure of all patents, patent documents and publications
25   cited herein are incorporated herein by reference as if individually incorporated. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described for variations obvious to one skilled in the art will be included within the invention
30   defined by the claims.

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WHAT IS CLAIMED IS:

1. An isolated and purified DNA molecule comprising a DNA segment
    encoding an oat acetyl CoA carboxylase, or a fragment thereof.

2. The DNA molecule of claim 1 which comprises a DNA segment
    comprising SEQ ID NO:23.

3. The DNA molecule of claim 1 or 2 further comprising a DNA molecule
    encoding an amino terminal chloroplast transit peptide, wherein the
    chloroplast transit peptide is operably linked to the DNA encoding the
    oat acetyl CoA carboxylase.

4. The DNA molecule of claim 1 which encodes a polypeptide comprising
    SEQ ID NO:24.

5. A method of imparting cyclohexanedione or aryloxyphenoxypropanoic
    acid herbicide tolerance to a plant tissue comprising:

    (a) introducing an expression cassette comprising a chimeric DNA
         molecule encoding an oat acetyl CoA carboxylase comprising
         SEQ ID NO:24, operably linked to a promoter functional in a
         plant cell into cells of a susceptible plant so as to yield
         transformed plant cells;

    (b) regenerating the transformed plant cells to provide a differentiated
         transformed plant; and

    (c) expressing the chimeric DNA molecule encoding the oat acetyl
         CoA carboxylase in the cells of the differentiated plant in an
         amount effective to render the plant tolerant to levels of a
         cyclohexanedione or an aryloxyphenoxypropanoic acid herbicide
         which are toxic to a susceptible plant which does not comprise the
         expression cassette.

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6.   The method of claim 5 wherein the chimeric DNA molecule encodes an
        acetyl CoA carboxylase, the activity of which is not inhibited by an
        amount of cyclohexanedione or aryloxyphenoxypropanoic acid herbicide
        that inhibits the activity of an acetyl CoA carboxylase which is sensitive
5   to inhibition by a cyclohexanedione or aryloxyphenoxypropanoic acid
        herbicide.

7.   The method of claim 5 wherein the chimeric DNA molecule is expressed
        in the cells at about a 2′ to about a 20-fold amount over that of a
10   cyclohexanedione or aryloxyphenoxypropanoic acid herbicide-sensitive
        acetyl CoA carboxylase.

8.   The method of claim 5 wherein the chimeric DNA molecule comprises a
        DNA sequence comprising SEQ ID NO:23.

15

9.   A transformed plant prepared by the method of claim 5.

10.   A transformed seed of the transformed plant of claim 9.

20   11.   A transformed monocot plant, which plant is tolerant to herbicides
           comprising:
           (a)   an acetyl CoA carboxylase gene that encodes an acetyl CoA
                  carboxylase the activity of which is inhibited by a
                  cyclohexanedione or aryloxyphenoxypropanoic acid herbicide,
25   and
           (b)   a recombinant DNA molecule encoding an oat acetyl CoA
                  carboxylase operably linked to a promoter functional in a plant,
                  wherein the recombinant DNA molecule is expressed in the plant
                  in an amount effective to confer tolerance to the levels of a
30   cyclohexanedione herbicide or an aryloxyphenoxypropanoic acid
                  herbicide which inhibit the activity of the acetyl CoA carboxylase
                  of step (a).

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12. A method for altering the oil content in a plant comprising:

    (a)   introducing an expression cassette comprising a recombinant DNA molecule encoding an oat acetyl CoA carboxylase operably linked to a promoter functional in a plant cell into the cells of a plant so as to yield transformed plant cells; and

    (b)   regenerating the transformed plant cells to provide a differentiated transformed plant, wherein the plant expresses the DNA molecule encoding the oat acetyl CoA carboxylase in cells of said plant so as to alter the oil content of the differentiated transformed plant cells relative to the oil content of cells of a corresponding untransformed plant.

13. The method according to claim 12, wherein the recombinant DNA molecule is expressed at about a 2- to about a 20-fold increase over the level of the native acetyl CoA carboxylase.

14. The method according to claim 13 wherein the oil content of the plant cells is increased about 1.2- to about 20-fold.

15. A transformed plant having an altered oil content in the plant cells comprising: a recombinant DNA molecule encoding an oat acetyl CoA carboxylase, wherein the recombinant DNA molecule is expressed in the cells of the plant in an amount of acetyl CoA carboxylase effective to alter the oil content of the plant cells relative to the oil content in the cells of a corresponding untransformed plant.

16. The transformed plant of claim 15 wherein the transformed plant has an increase in oil content of about 1.2- to about 20-fold in its leaves, seeds, or fruit above that present in a corresponding untransformed plant.

17. A transformed plant prepared by the method of claim 12.

18. A seed of the transformed plant of claim 17.

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19.   The seed of claim 18 wherein the oil content is increased in the
       endosperm.

20.   A method of introducing an exogenous oat acetyl CoA carboxylase gene
       into a host cell comprising:
       (a)   introducing to host cells *in vitro* an expression cassette
              comprising a DNA molecule encoding an oat acetyl CoA
              carboxylase operably linked to a promoter functional in the host
              cell so as to yield transformed cells; and
       (b)   identifying transformed host cells which express the DNA
              molecule.

21.   The method of claim 20 wherein the host cells are plant cells.

22.   The method of claim 21 wherein the plant cells can be regenerated into a
       differentiated plant.

23.   The method of claim 21 wherein the plant cells are monocot cells.

24.   The method of claim 21 wherein the plant cells are dicot cells.

25.   The method of claim 12, wherein the DNA molecule encoding the oat
       acetyl CoA carboxylase is introduced into plant cells by a method
       selected from the group consisting of electroporation, microinjection,
       protoplast transformation, microprojectile bombardment, and liposomal
       encapsulation, *Agrobacterium*-mediated transformation.

26.   A method for altering the oil content in a plant comprising:
       (a)   introducing an expression cassette comprising a recombinant
              DNA molecule encoding an antisense oat acetyl CoA carboxylase
              transcript operably linked to a promoter functional in a plant cell
              into the cells of a plant so as to yield transformed plant cells; and

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(b)   regenerating the transformed plant cells to provide a differentiated
        transformed plant, wherein the transcript is expressed in the cells
        of the differentiated transformed plant so as to alter the oil content
        of the plant cells relative to the cells of a corresponding
        untransformed plant.

27.   A transformed plant having an altered oil content in the plant cells
        comprising: a recombinant DNA molecule encoding an antisense oat
        acetyl CoA carboxylase transcript wherein the transcript is expressed in
        the cells of the plant so as to alter the oil content of the plant cells relative
        to the cells of a corresponding untransformed plant.

28.   The method of claim 12 or 26 wherein the recombinant DNA molecule
        further comprises a DNA segment encoding a chloroplast transit peptide
        which is operably linked to the DNA molecule encoding the acetyl CoA
        carboxylase.

29.   The transformed plant of claim 15 or 27 wherein the recombinant DNA
        molecule further comprises a DNA segment encoding a chloroplast
        transit peptide which is operably linked to the DNA molecule encoding
        the acetyl CoA carboxylase.

30.   The method of claim 12 or 26 wherein the recombinant DNA molecule
        further comprises a DNA segment comprising a endosperm-specific
        promoter.

31.   The plant of claim 15 or 27 wherein the recombinant DNA molecule
        further comprises a DNA segment comprising a endosperm-specific
        promoter.

32.   The method of claim 12 or 26 wherein the cells are monocot plant cells.

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33.   The method of claim 32 wherein the cells are wheat, rice, maize, barley, millet, sorghum, sugarcane, rye, oat or timothy cells.

34.   The method of claim 12 or 26 wherein the cells are dicot plant cells.

5

35.   The method of claim 34 wherein the cells are soybean, rape, sunflower, tobacco, Arabidopsis, petunia, pea, Canola, bean, tomato, bean, potato, lettuce, spinach, alfalfa, cotton or carrot.

10   36.   The method of claim 12, 20, or 25 wherein the DNA molecule encodes a polypeptide comprising SEQ ID NO:24.

37.   The plant of claim 11, 15, or 27 wherein the DNA molecule encodes a polypeptide comprising SEQ ID NO:24.

15

38.   An expression cassette comprising an endosperm-specific transcriptional control region operably linked to a DNA encoding a plant ACCase, or a biologically active subunit thereof.

20   39.   The expression cassette of claim 38 wherein the endosperm-specific transcriptional control region comprises Dy10.

40.   The plant of claim 15 which has increased lipid content, lipid biosynthesis or lipid deposition in groat.

25

41.   The plant of claim 27 which has decreased lipid content, lipid biosynthesis or lipid deposition in groat.

42.   The plant of claim 15 which has increased storage of seed lipid in
30   endosperm.

43.   The plant of claim 27 which has decreased storage of seed lipid in endosperm.

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44.   A method to identify an oat plant having altered groat oil content, comprising:

    (a)   contacting a probe comprising at least a portion of a nucleic acid sequence encoding oat acetyl CoA carboxylase with a nucleic

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        acid sample from a plant in an amount sufficient to form complexes, wherein the sample comprises genomic nucleic acid; and

    (b)   detecting or determining the amount of complex formation.

10   45.   A method to identify a plant having altered oil content, β-glucan content, seed weight, starch content or protein content, comprising:

    (a)   contacting a probe comprising at least a portion of a nucleic acid sequence encoding oat acetyl CoA carboxylase with a sample from a plant in an amount sufficient to form complexes, wherein

15

        the sample comprises genomic nucleic acid; and

    (b)   detecting or determining the amount of complex formation.

46.   The method of claim 45 wherein the plant is an oat, maize or wheat plant.

20   47.   A method for marker-assisted selection of plants having desired properties, comprising:

    (a)   contacting a probe comprising at least a portion of a nucleic acid sequence encoding oat acetyl CoA carboxylase with a sample from a plant in an amount sufficient to form complexes, wherein

25

        the sample comprises genomic nucleic acid; and

    (b)   detecting or determining the amount of complex formation.

48.   An isolated and purified nucleic acid segment associated with altered oil content or composition in monocots.

30

49.   A method to map a restriction fragment length polymorphism in plants, comprising:

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    (a)   contacting a probe comprising at least a portion of a nucleic acid

sequence encoding oat acetyl CoA carboxylase with a sample

from a plant in an amount sufficient to form complexes, wherein

the sample comprises genomic nucleic acid; and

5   (b)   detecting or determining the amount of complex formation.

50.   The method of claim 44, 45, 47 or 49 wherein the genomic nucleic acid is

from oat, maize, rice or wheat.

FATTY ACID BIOSYNTHESIS
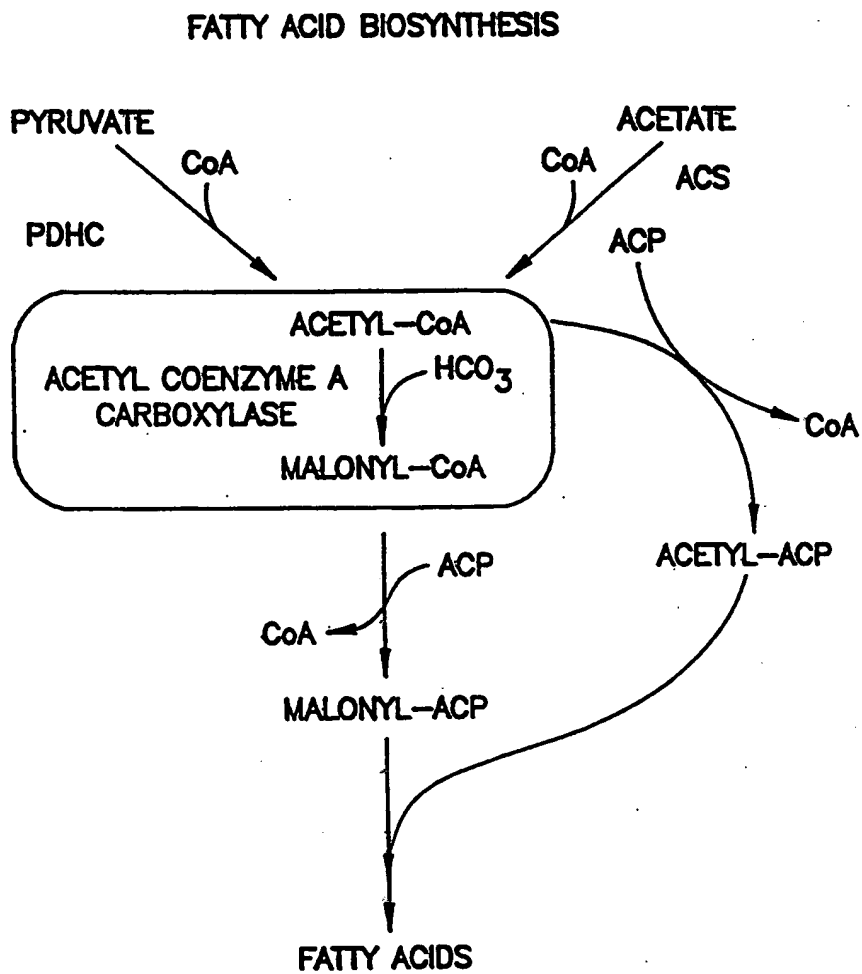
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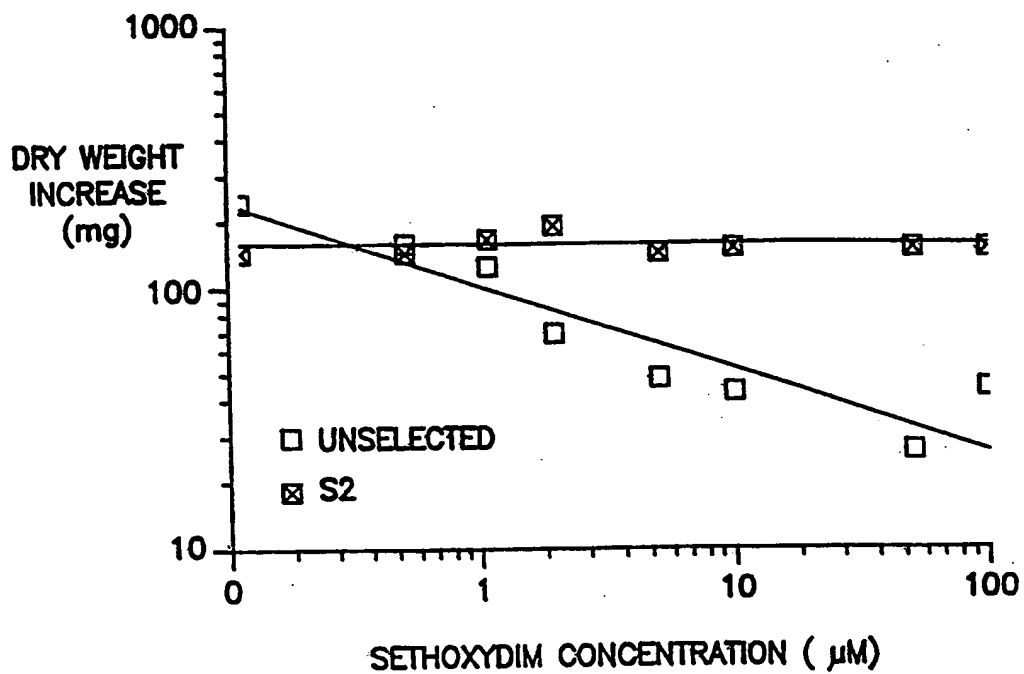
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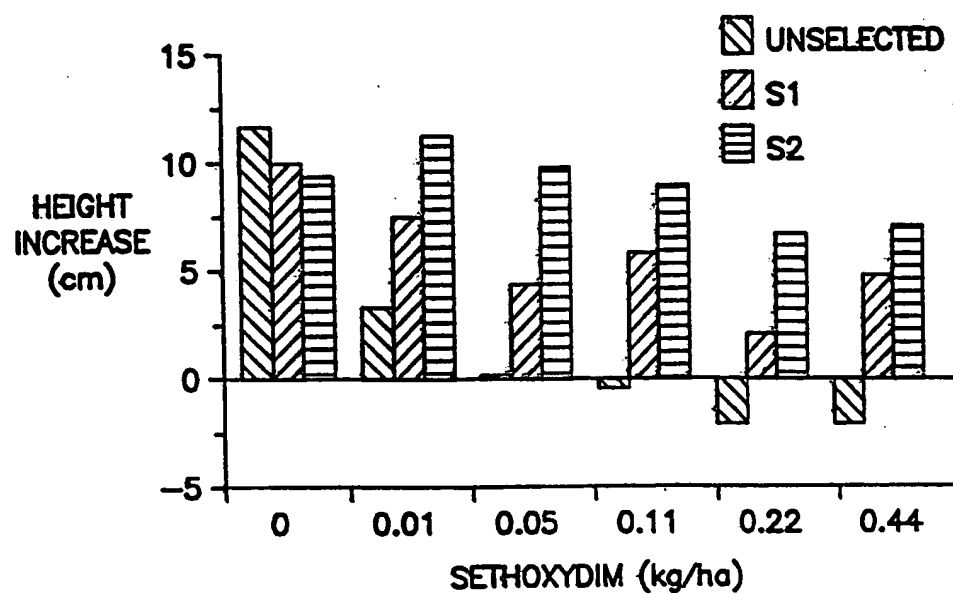
FIG. 3
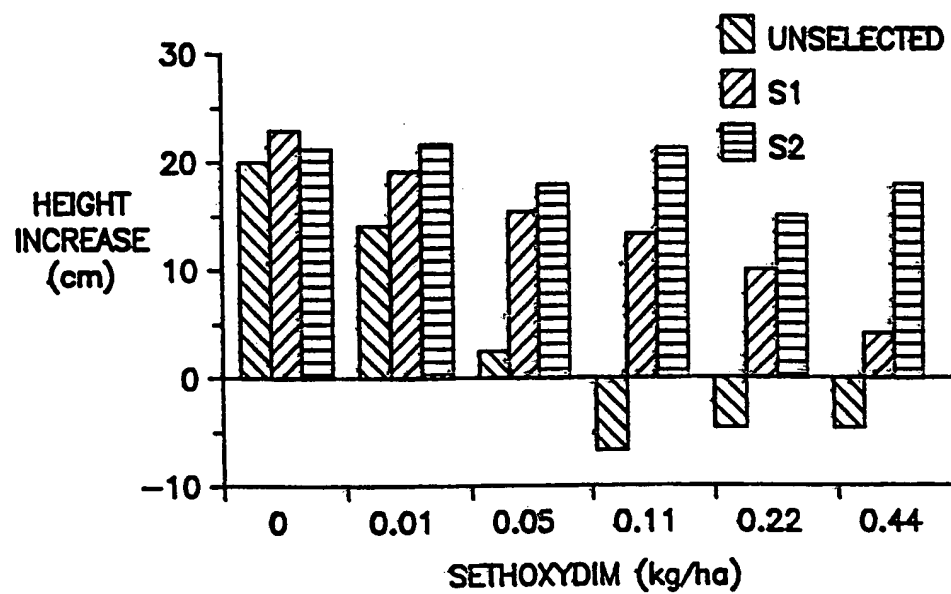
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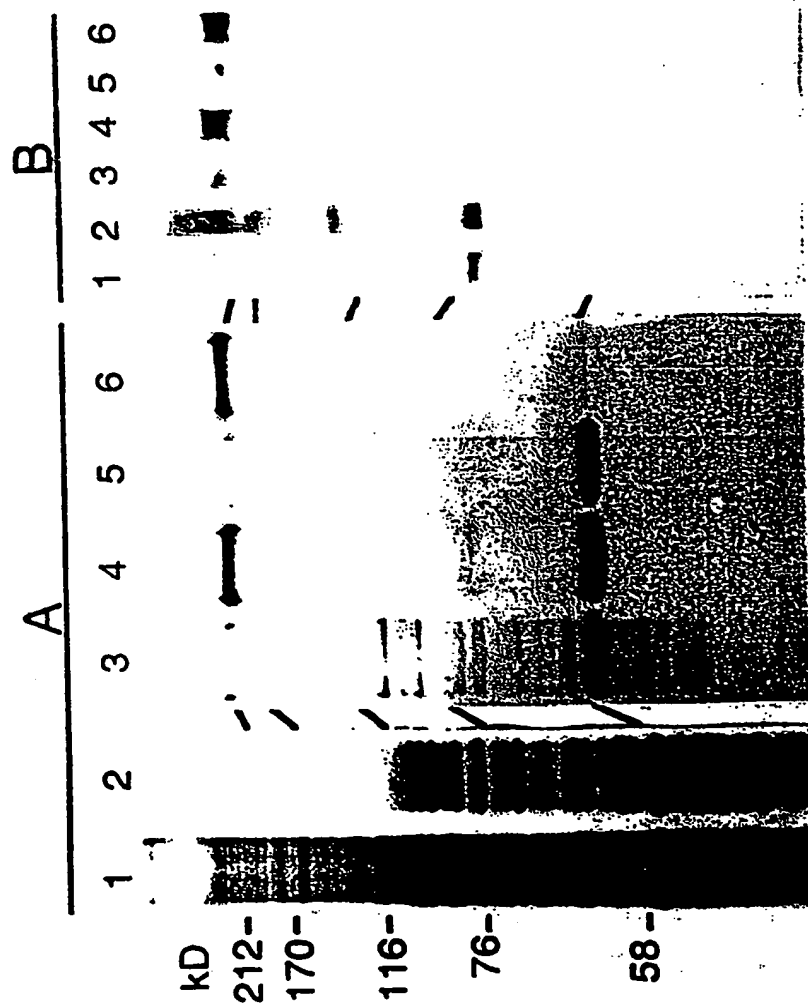
FIG.5
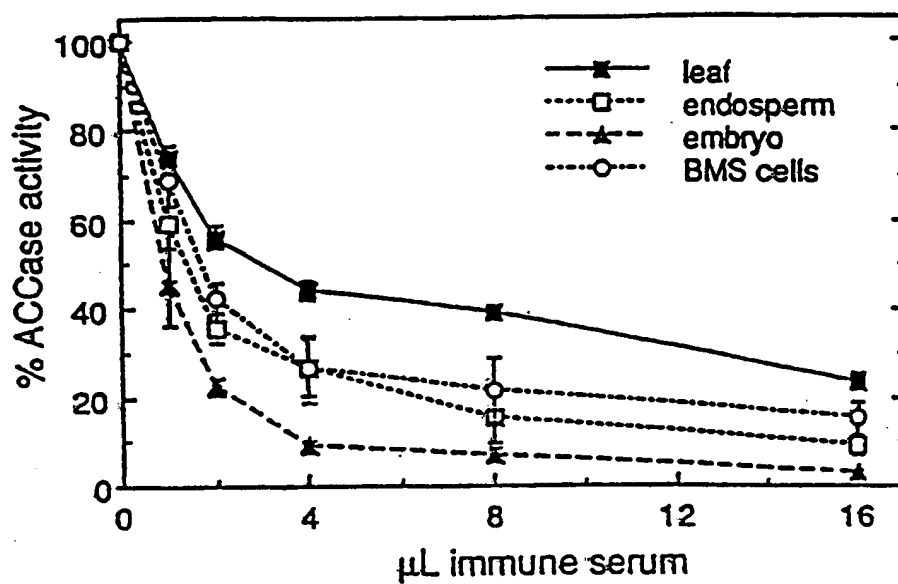
FIG. 6
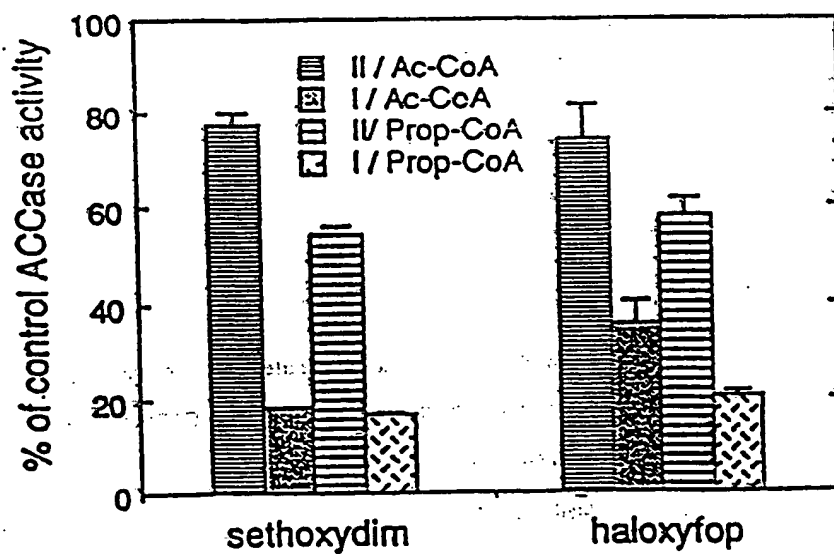
FIG. 7
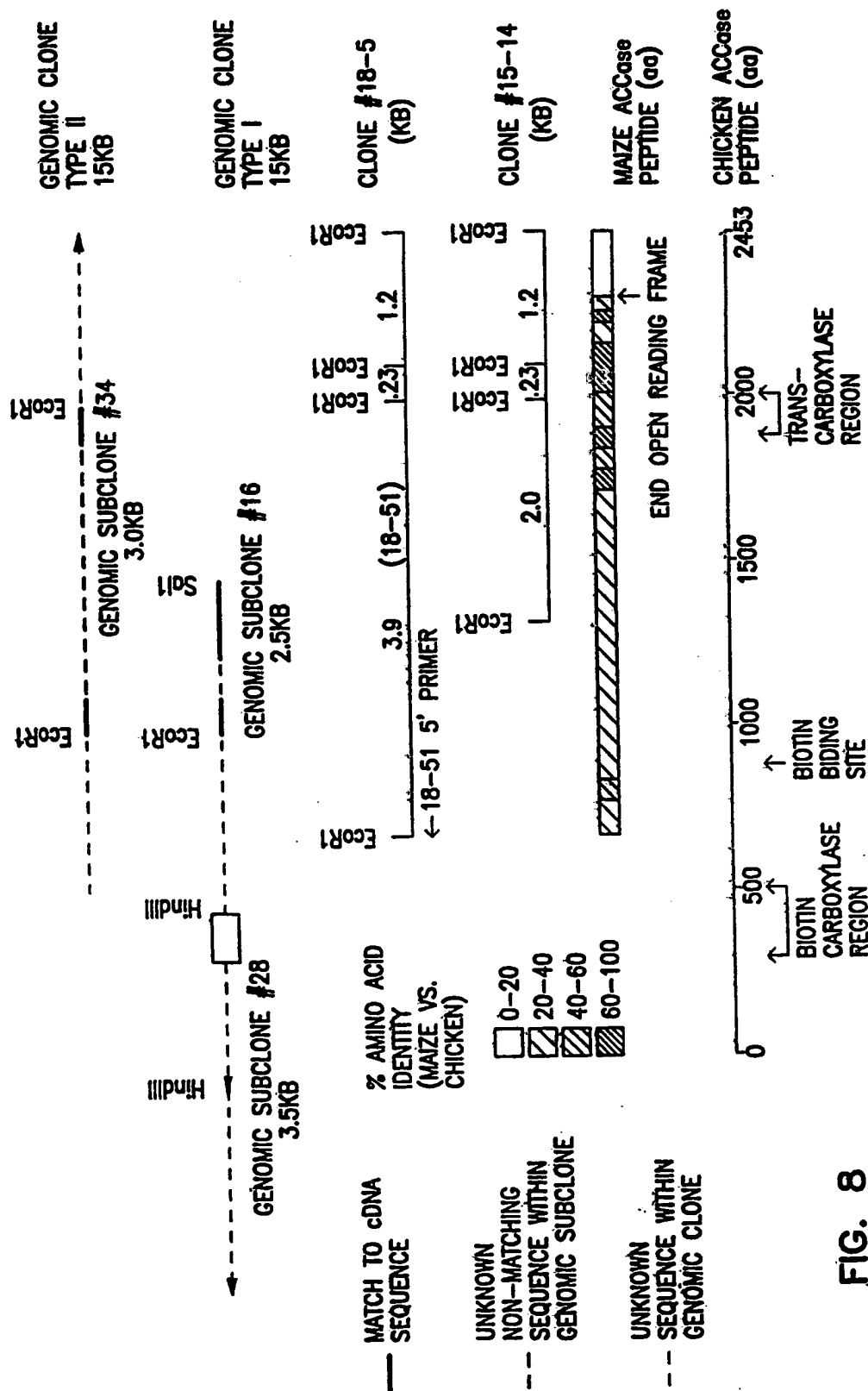
FIG. 8
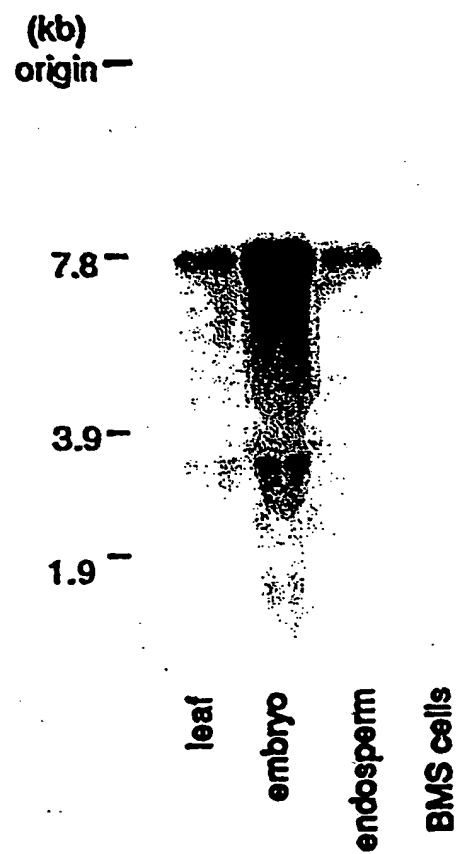
FIG.9

AGA GAT GAA GCT CGC ATG CCA ATG CGC CAC ACA TTC CTC TGG TTG GAT
GAC AAG AGT TGT TAT GAA GAA GAG CAG ATT CTC CGG CAT GTG GAG CCT
CCC CTC TCT ACA CTT CTT GAA TTG GAT AAG TTG AAG GTG AAA GGA TAC
AAT GAA ATG AAG TAT ACT CCT TCG CGT GAC CGC CAA TGG CAT ATC TAC
ACA CTA AGA AAT ACT GAA AAC CCC AAA ATG TTG CAT AGG GTG TTT TTC
CGA ACT ATT GTC AGG CAA CCC AAT GCA GGC AAC AAG TTT AGA TCG GCT
CAG ATC AGC GAC GCN AAG GTA GGA TGT CCC GAA GAA TCT CTT TCA TTT
ACA TCA AAT AGC ATC TTA AGA TCA TTG ATG ACT GCT ATT GAA GAA TTA
GAG CTT CAT GCA ATT AGG ACA GGT CAT TCT CAC ATG TAT TTG TGC ATA
CTG AAA GAG CAA AAG CTT CTT GAC CTC ATT CCA TTT TCA GGG AGT ACA
ATT GTT GAT GTT GGC CAA GAT GAA GCT ACC GCT TGT TCA CTT TTA AAA
TCA ATG GCT TTG AAG ATA CAT GAG CTT GTT GGT GCA AGG ATG CAT CAT
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CCT GCA AGT GGT ACC TGG AGA GTT GTA ACT ACA AAT GTT ACT GGT CAC
ACC TGC ACC ATT GAT ATA TAC CGA GAA GTG GAG GAA ATA GAA TCA CAG
AAG TTA GTG TAC CAT TCA GCC AGT TCG TCA GCT GGA CCA TTG CAT GGT
GTT GCA CTG AAT AAT CCA TAT CAA CCT TTG AGT GTG ATT GAT CTA AAG
CGC TGC TCT GCT AGG AAC AAC AGA ACA ACA TAT TGC TAT GAT TTT CCG
CTG GCC TTT GAA ACT GCA CTG CAG AAG TCA TGG CAG TCC AAT GGC TCT
ACT GTT TCT GAA GGC AAT GAA AAT AGT AAA TCC TAC GTG AAG GCA ACT
GAG CTA GTG TTT GCT GAA AAA CAT GGG TCC TGG GGC ACT CCT ATA ATT
CCG ATG GAA CGC CCT GCT GGG CTC AAC GAC ATT GGT ATG GTC GCT TGG
ATC ATG GAG ATG TCA ACA CCT GAA TTT CCC AAT GGC AGG CAG ATT ATT
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GAA GAT GCA TTT TTT GAA ACT GTC ACT AAC CTG GCT TGC GAA AGG AAA
CTT CCT CTT ATA TAC TTG GCA GCA AAC TCT GGT GCT AGG ATT GGC ATA
GCT GAT GAA GTA AAA TCT TGC TTC CGT GTT GGA TGG TCT GAC GAA GGC
AGT CCT GAA CGA GGG TTT CAG TAC ATC TAT CTG ACT GAA GAA GAC TAT
GCT CGC ATT AGC TCT TCT GTT ATA GCA CAT AAG CTG GAG CTA GAT AGT
GGT GAA ATT AGG TGG ATT ATT GAC TCT GTT GTG GGC AAG GAG GAT GGG
CTT GGT GTC GAG AAC ATA CAT GGA AGT GCT GCT ATT GCC AGT GCT TAT
TCT AGG GCA TAT GAG GAG ACA TTT ACA CTT ACA TTT GTG ACT GGG CGG
ACT GTA GGA ATA GGA GCT TAT CTT GCT CGA CTT GGT ATA CGG TGC ATA
CAG CGT CTT GAC CAG CCT ATT ATT TTA ACA GGG TTT TCT GCC CTG AAC
AAG CTC CTT GGG CGG GAA GTG TAC AGC TCC CAC ATG CAG CTT GGT GGT
CCT AAG ATC ATG GCG ACC AAT GGT GTT GTC CAC CTC ACT GTT CCA GAT

FIG. 10

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GTC CTT GAA GGT GTT TCC AAT ATA TTG AGG TGG CTC AGC TAT GTT CCT
GCA AAC ATT GGT GGA CCT CTT CCT ATT ACC AAA CCT CTG GAC CCT CCA
GAC AGA CCT GTT GCT TAC ATC CCT GAG AAC ACA TGC GAT CCA CGT GCA
GCT ATC TGT GGT GTA GAT GAC AGC CAA GGG AAA TGG TTG GGT GGT ATG
TTT GAC AAA GAC AGC TTT GTG GAG ACA TTT GAA GGA TGG GCA AAA ACA
GTG GTT ACT GGC AGA GCA AAG CTT GGA GGA ATT
```

FIG. 10 (continued)
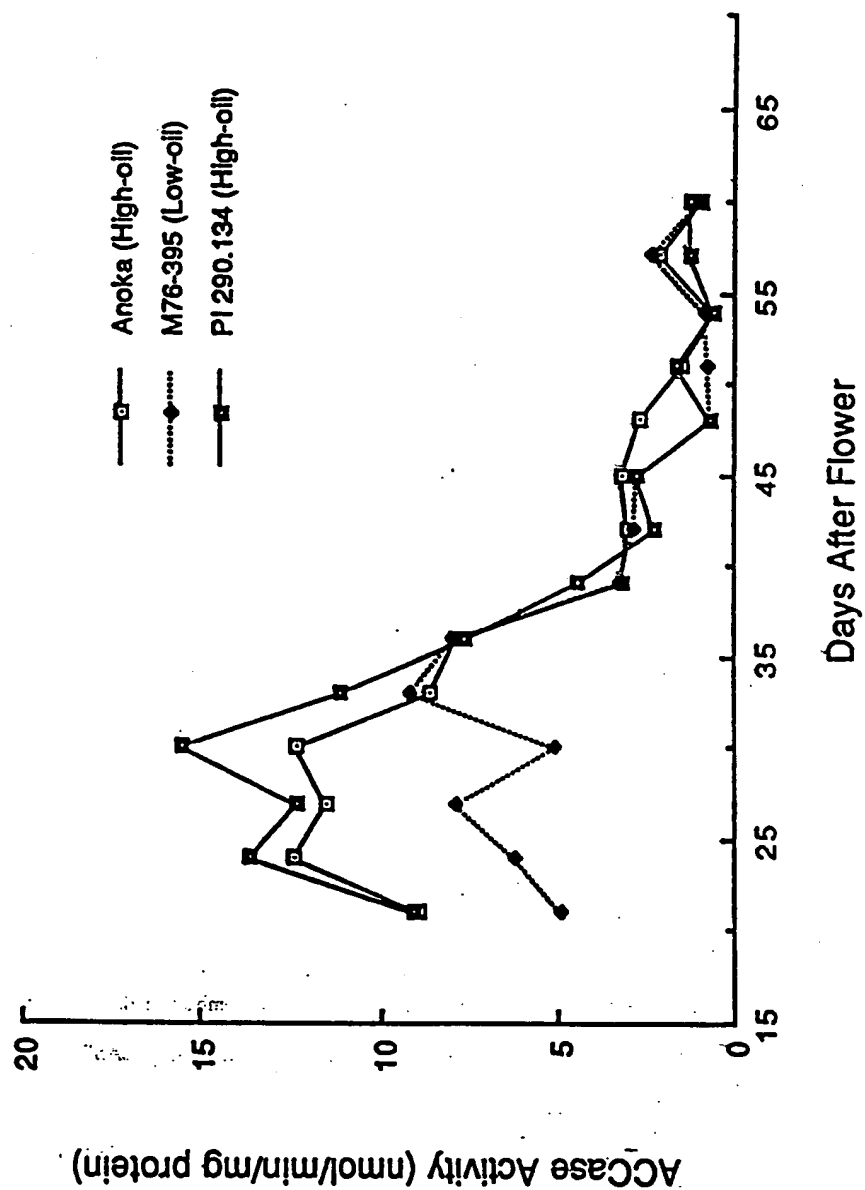
FIG. 11

ACCase A cloning strategy and position of start and stop codons
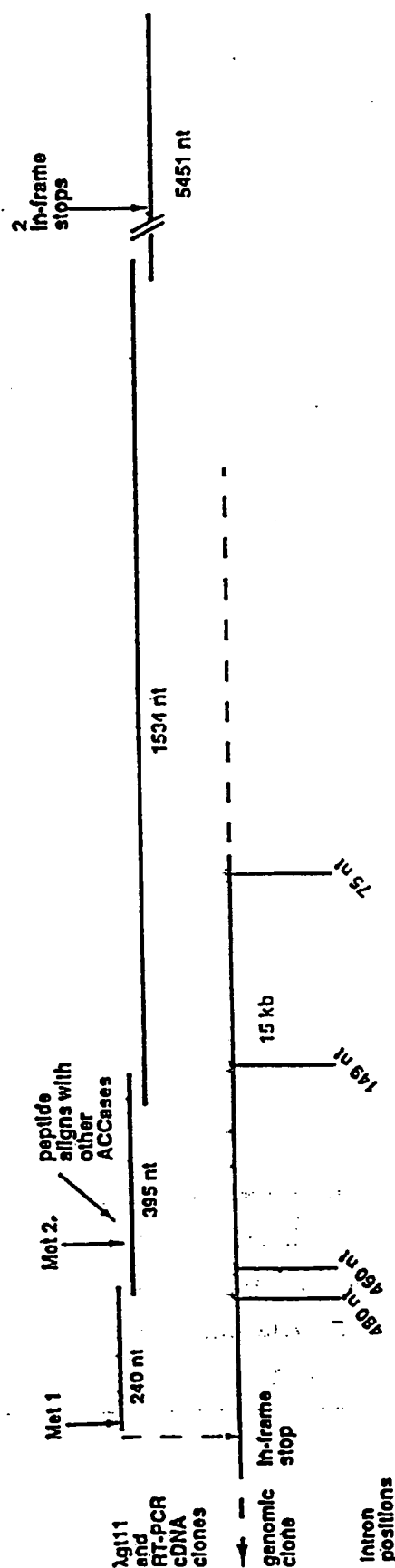
FIG. 12

```
   1   GGTCTTCAAT TGTGCTGTCT GGGCCACGGA ACGACAATGT CACAGCTTGG
  51   ATTAGCCGCA GCTGCCTCAA AGGCCTTGCC ACTACTCCCT AATCGCCAGA
 101   GAAGTTCAGC TGGGACTACA TTCTCATCAT CTTCATTATC GAGGCCCTTA
 151   AACAGAAGGA AAAGCCATAC TCGTTCACTC CGTGATGGCG GAGATGGGGT
 201   ATCAGATGCC AAAAAGCACA GCCAGTCTGT TCGTCAAGGT CTTGCTGGCA
 251   TTATCGACCT CCCAAGTGAG GCACCTTCCG AAGTGGATAT TTCACATGGA
 301   TCTGAGGATC CTAGGGGGCC AACAGATTCT TATCAAATGA ATGGGATTAT
 351   CAATGAAACA CATAATGGAA GACATGCCTC AGTGTCCAAG GTTGTTGAAT
 401   TTTGTGCGGC ACTAGGTGGC AAAACACCAA TTCACAGTAT ATTAGTGGCC
 451   AACAATGGAA TGGCAGCAGC AAAATTTATG AGGAGTGTCC GGACATGGGC
 501   TAATGATACT TTTGGATCTG AGAAGGCAAT TCAACTCATA GCTATGGCAA
 551   CTCCGGAAGA CATGAGGATA AATGCAGAAC ACATTAGAAT TGCTGACCAA
 601   TTCGTAGAGG TGCCTGGTGG AACAAACAAT AATAACTACG CCAATGTTCA
 651   ACTCATAGTG GGGATGGCAC AAAAACTAGG TGTTTCTGCT GTTTGGCCTG
 701   GTTGGGGTCA TGCTTCTGAG AATCCTGAAC TGCCAGATGC ATTGACCGCA
 751   AAAGGGATCG TTTTTCTTGG CCCACCTGCA TCATCAATGA ATGCTTTGGG
 801   AGATAAGGTC GGCTCAGCTC TCATTGCTCA AGCAGCCGGG GTCCCAACTC
 851   TTGCTTGGAG TGGATCACAT GTTGAAGTTC CATTAGAGTG CTGCTTAGAC
 901   GCGATACCTG AGGAGATGTA TAGAAAAGCT TGCGTTACTA CCACAGAGGA
 951   AGCAGTTGCA AGTTGTCAAG TGGTTGGTTA TCCTGCCATG ATTAAGGCAT
1001   CCTGGGGAGG TGGTGGTAAA GGAATAAGAA AGGTTCATAA TGATGATGAG
1051   GTTAGAGCGC TGTTTAAGCA AGTACAAGGT GAAGTCCCTG GCTGCCCAAT
1101   ATTTGTCATG AGGCTTGCAT CCCAGAGTCG GCATCTTGAA GTTCAGTTGC
1151   TTTGTGATCA ATATGGTAAT GTAGCAGCAC TTCACAGTCG TGATTGCAGT
1201   GTGCAACGGC GACACCAGAA GATTATTGAA GAAGGTCCAG TTACTGTTGC
1251   TCCTCGTGAG ACAGTTAAAG CACTTGAGCA GGCAGCAAGG AGGCTTGCTA
1301   AGGCTGTGGG TTATGTTGGT GCTGCTACTG TTGAGTATCT TTACAGCATG
1351   GAAACTGGAG ACTACTATTT TCTGGAACTT AATCCCGAC TACAGGTTGA
1401   GCATCCAGTC ACTGAGTGGA TAGCTGAAGT GAATCTGCCT GCAGCTCAAG
1451   TTGCTGTTGG AATGGGCATA CCTCTTTGGC AGATTCCAGA AATCAGACGT
1501   TTCTATGGAA TGGACTATGG AGGAGGGTAT GACATTTGGA GGAAAACAGC
1551   AGCTCTTGCT ACACCATTTA ATTTTGATGA AGTAGATTCT CAATGGCCAA
1601   AGGGCCATTG TGTAGCAGTT AGAATTACTA GTGAGGACCC AGATGATGGT
1651   TTCAAACCTA CTGGTGGGAA AGTGAAGGAG ATAAGTTTTA AAAGCAAGCC
1701   TAATGTTTGG GCCTACTTCT CAGTAAAGTC TGGTGGAGGC ATTCATGAAT
1751   TTGCTGATTC TCAGTTTGGA CATGCTTTTG CATATGGACT CTCTAGACCA
```

FIG. 13

```
1801   GCAGCTATAA CAAACATGTC TCTTGCATTA AAAGAGATTC AGATTCGTGG
1851   AGAAATTCAT TCAAATGTTG ATTACACAGT TGACCTCTTA AACGCTTCAG
1901   ACTTCAGAGA AAACAAGATC CACACTGGTT GGCTGGATAC AAGAATAGCT
1951   ATGCGTGTTC AAGCTGAGAG GCCCCCATGG TATATCTCAG TGGTTGGAGG
2001   TGCTTTATAT AAAACAGTAA CCACCAATGC AGCCACTGTT TCTGAATATG
2051   TTAGTTATCT CACCAAGGGC CATATTCCAC CAAAGCATAT ATCCCTTGTC
2101   AATTCTACAG TTAATTTGAA TATAGAAGGG AGCAAATACA CAATTGAAAC
2151   TGTAAGGACT GGACATGGTA GCTACAGGTT GAGAATGAAT GATTCAACAG
2201   TTGAAGCGAA TGTACAATCT TTATGTGATG GTGGCCTCTT AATGCAGTTG
2251   GATGGAAACA GCCATGTAAT TTATGCAGAA GAAGAAGCTG GTGGTACACG
2301   GCTTCAGATT GATGGAAAGA CATGTTTATT GCAGAATGAC CATGATCCAT
2351   CGAAGTTATT AGCTGAGACA CCCTGCAAAC TTCTTCGTTT CTTGGTTGCT
2401   GATGGTGCTC ATGTTGATGC GGATGTACCA TACGCGGAAG TTGAGGTTAT
2451   GAAGATGTGC ATGCCTCTCT TGTCACCTGC TTCTGGTGTC ATTCATTGTA
2501   TGATGTCTGA GGGCCAGGCA TTGCAGGCTG GTGATCTTAT AGCAAGGTTG
2551   GATCTTGATG ACCCTTCTGC TGTGAAAAGA GCTGAGCCAT TTGATGGAAT
2601   ATTTCCACAA ATGGAGCTCC CTGTTGCTGT CTCTAGTCAA GTACACAAAA
2651   GATATGCTGC AAGTTTGAAT GCTGCTCGAA TGGTCCTTGC AGGATATGAG
2701   CACAATATTA ATGAAGTCGT TCAAGATTTG GTATGCTGCC TGGACAACCC
2751   TGAGCTTCCT TTCCTACAGT GGGATGAACT TATGTCTGTT CTAGCAACGA
2801   GGCTTCCAAG AAATCTCAAG AGTGAGTTAG AGGATAAATA CAAGGAATAC
2851   AAGTTGAATT TTTACCATGG AAAAAACGAG GACTTTCCAT CCAAGTTGCT
2901   AAGAGACATC ATTGAGGAAA ATCTTTCTTA TGGTTCAGAG AAGGAAAAGG
2951   CTACAAATGA GAGGCTTGTT GAGCCTCTTA TGAAGCTACT GAAGTCATAT
3001   GAGGGTGGGA GAGAGAGCCA TGCACATTTT GTTGTCAAGT CTCTTTTCGA
3051   GGAGTATCTT ACAGTGGAAG AACTTTTTAG TGATGGCATT CAGTCTGACG
3101   TGATTGAAAC ATTGCGGCAT CAGCACAGTA AAGACCTGCA GAAGGTTGTA
3151   GACATTGTGT TGTCTCACCA GGGTGTGAGG AACAAAGCTA AGCTTGTAAC
3201   GGCACTTATG GAAAAGCTGG TTTATCCAAA TCCTGGTGGT TACAGGGATC
3251   TGTTAGTTCG CTTTTCTTCC CTCAATCATA AAAGATATTA TAAGTTGGCC
3301   CTTAAAGCAA GTGAACTTCT TGAACAAACC AAACTAAGTG AACTCCGTGC
3351   AAGCGTTGCA AGAAGCCTTT CGGATCTGGG GATGCATAAG GGAGAAATGA
3401   GTATTAAGGA TAACATGGAA GATTTAGTCT CTGCCCCATT ACCTGTTGAA
3451   GATGCTCTGA TTTCTTTGTT TGATTACAGT GATCGAACTG TTCAGCAGAA
3501   AGTGATTGAG ACATACATAT CACGATTGTA CCAGCCTCAT CTTGTAAAGG
3551   ATAGCATCCA AATGAAATTC AAGGAATCTG GTGCTATTAC TTTTTGGGAA
```

FIG. 13 (continued)

```
3601  TTTTATGAAG GGCATGTTGA TACTAGAAAT GGACATGGGG CTATTATTGG
3651  TGGGAAGCGA TGGGGTGCCA TGGTCGTTCT CAAATCACTT GAATCTGCGT
3701  CAACAGCCAT TGTGGCTGCA TTAAAGGATT CGGCACAGTT CAACAGCTCT
3751  GAGGGCAACA TGATGCACAT TGCATTATTG AGTGCTGAAA ATGAAAGTAA
3801  TATAAGTGGA ATAAGCAGTG ATGATCAAGC TCAACATAAG ATGGAAAAGC
3851  TTAGCAAGAT ACTGAAGGAT ACTAGCGTTG CAAGTGATCT CCAAGCTGCT
3901  GGTTTGAAGG TTATAAGTTG CATTGTTCAA AGAGATGAAG CTCGCATGCC
3951  AATGCGCCAC ACATTCCTCT GGTTGGATGA CAAGAGTTGT TATGAAGAAG
4001  AGCAGATTCT CCGGCATGTG GAGCCTGCCC TCTCTACACT TCTTGAATTG
4051  GATAAGTTGA AGGTGAAAGG ATACAATGAA ATGAAGTATA CTCCTTCGCG
4101  TGACCGCCAA TGGCATATCT ACACACTAAG AAATACTGAA AACCCCAAAA
4151  TGTTGCATAG GGTGTTTTTC CGAACTATTG TCAGGCAACC CAATGCAGGC
4201  AACAAGTTTA GATCGGCTCA GATCAGCGAC GCTGAGGTAG GATGTGGCGA
4251  AGAATCTCTT TCATTTACAT CAAATAGCAT CTTAAGATCA TTGATGACTG
4301  CTATTGAAGA ATTAGAGCTT CATGCAATTA GGACAGGTCA TTCTCACATG
4351  TATTTGTGCA TACTGAAAGA GCAAAAGCTT CTTGACCTCA TTCCATTTTC
4401  AGGGAGTACA ATTGTTGATG TTGGCCAAGA TGAAGCTACC GCTTGTTCAC
4451  TTTTAAAATC AATGGCTTTG AAGATACATG AGCTTGTTGG TGCAAGGATG
4501  CATCATCTGT CTGTATGCCA GTGGGAGGTG AAACTCAAGT TGGACTGTGA
4551  TGGCCCTGCA AGTGGTACCT GGAGAGTTGT AACTACAAAT GTTACTGGTC
4601  ACACCTGCAC CATTGATATA TACCGAGAAG TGGAGGAAAT AGAATCACAG
4651  AAGTTAGTGT ACCATTCAGC CAGTTCGTCA GCTGGACCAT TGCATGGTGT
4701  TGCACTGAAT AATCCATATC AACCTTTGAG TGTGATTGAT CTAAAGCGCT
4751  GCTCTGCTAG GAACAACAGA ACAACATATT GCTATGATTT TCCGCTGGCC
4801  TTTGAAACTG CACTGCAGAA GTCATGGCAG TCCAATGGCT CTACTGTTTC
4851  TGAAGGCAAT GAAAATAGTA AATCCTACGT GAAGGCAACT GAGCTAGTGT
4901  TTGCTGAAAA ACATGGGTCC TGGGGCACTC CTATAATTCC GATGGAACGC
4951  CCTGCTGGGC TCAACGACAT TGGTATGGTC GCTTGGATCA TGGAGATGTC
5001  AACACCTGAA TTTCCCAATG GCAGGCAGAT TATTGTTGTA GCAAATGATA
5051  TCACTTTCAG AGCTGGATCA TTTGGCCCAA GGGAAGATGC ATTTTTTGAA
5101  ACTGTCACTA ACCTGGCTTG CGAAAGGAAA CTTCCTCTTA TATACTTGGC
5151  AGCAAACTCT GGTGCTAGGA TTGGCATAGC TGATGAAGTA AAATCTTGCT
5201  TCCGTGTTGG ATGGTCTGAC GAAGGCAGTC CTGAAGGAGG GTTTCAGTAC
5251  ATCTATCTGA CTGAAGAAGA CTATGCTCGC ATTAGCTCTT CTGTTATAGC
5301  ACATAAGCTG GAGCTAGATA GTGGTGAAAT TAGGTGGATT ATTGACTCTG
5351  TTGTGGGCAA GGAGGATGGG CTTGGTGTCG AGAACATACA TGGAAGTGCT
```

FIG. 13 (continued)

```
5401  GCTATTGCCA  GTGCTTATTC  TAGGGCATAT  GAGGAGACAT  TTACACTTAC
5451  ATTTGTGACT  GGGCGGACTG  TAGGAATAGG  AGCTTATCTT  GCTCGACTTG
5501  GTATACGGTG  CATACAGCGT  CTTGACCAGC  CTATTATTTT  AACAGGGTTT
5551  TCTGCCCTGA  ACAAGCTCCT  TGGGCGGGAA  GTGTACAGCT  CCCACATGCA
5601  GCTTGGTGGT  CCTAAGATCA  TGGCGACCAA  TGGTGTTGTC  CACCTCACTG
5651  TTCCAGATGT  CCTTGAAGGT  GTTCCAATA  TATTGAGGTG  GCTCAGCTAT
5701  GTTCCTGCAA  ACATTGGTGG  ACCTCTTCCT  ATTACCAAAC  CTCTGGACCC
5751  TCCAGACAGA  CCTGTTGCTT  ACATCCCTGA  GAACACATGC  GATCCACGTG
5801  CAGCTATCTG  TGGTGTAGAT  GACAGCCAAG  GGAAATGGTT  GGGTGGTATG
5851  TTTGACAAAG  ACAGCTTTGT  GGAGACATTT  GAAGGATGGG  CAAAAACAGT
5901  GGTTACTGGC  AGAGCAAAGC  TTGGAGGAAT  TCCTGTGGGC  GTCATAGCTG
5951  TGGAGACACA  GACCATGATG  CAGATCATCC  CTGCTGATCC  AGGTCAGCTT
6001  GATTCCCATG  AGCGATCTGT  CCCTCGTGCT  GGACAAGTGT  GGTTCCCAGA
6051  TTCTGCAACC  AAGACCGCTC  AGGCATTATT  AGACTTCAAC  CGTGAAGGAT
6101  TGCCTCTGTT  CATCCTGGCT  AATTGGAGAG  GCTTCTCTGG  TGGACAAAGA
6151  GATCTCTTTG  AAGGAATTCT  TCAGGCTGGG  TCAACAATTG  TCGAGAACCT
6201  TAGGACATAT  AATCAGCCTG  CTTTTGTGTA  CATTCCTATG  GCTGGAGAGC
6251  TTCGTGGAGG  AGCTTGGGTT  GTGGTCGATA  GCAAAATAAA  TCCAGACCGC
6301  ATTGAGTGTT  ATGCTGAAAG  GACTGCCAAA  GGTAATGTTC  TGGAACCTCA
6351  AGGGTTAATT  GAAATCAAGT  TCAGGTCAGA  GGAACTCCAA  GACTGTATGG
6401  GTAGGCTTGA  CCCAGAGTTG  ATAAATCTGA  AAGCAAAACT  CCAAGATGTA
6451  AATCATGGAA  ATGGAAGTCT  ACCAGACATA  GAAGGGATTC  GGAAGAGTAT
6501  AGAAGCACGT  ACGAAACAGT  TGCTGCCTTT  ATATACCCAG  ATTGCAATAC
6551  GGTTTGCTGA  ATTGCATGAT  ACTTCCCTAA  GAATGGCAGC  TAAAGGTGTG
6601  ATTAAGAAAG  TTGTAGACTG  GGAAGAATCA  CGCTCGTTCT  TCTATAAAAG
6651  GCTACGGAGG  AGGATCGCAG  AAGATGTTCT  TGCAAAAGAA  ATAAGGCAGA
6701  TAGTCGGTGA  TAAATTTACG  CACCAATTAG  CAATGGAGCT  CATCAAGGAA
6751  TGGTACCTTG  CTTCTCAGGC  CACAACAGGA  AGCACTGGAT  GGGATGACGA
6801  TGATGCTTTT  GTTGCCTGGA  AGGACAGTCC  TGAAAACTAC  AAGGGGCATA
6851  TCCAAAAGCT  TAGGGCTCAA  AAAGTGTCTC  ATTCGCTCTC  TGATCTTGCT
6901  GACTCCAGTT  CAGATCTGCA  AGCATTCTCG  CAGGGTCTTT  CTACGCTATT
6951  AGATAAGATG  GATCCCTCTC  AGAGAGCGAA  GTTTGTTCAG  GAAGTCAAGA
7001  AGGTCCTTGA  TTGATGATAC  CAACACATCC  AACACAATGT  GTGCATGTCA
7051  CATCTTTTTG  TTCTAGTACA  TACATAGAAG  GATATTGCTT  GGTCTTGATT
7101  GATCATGTCT  GATTTAAGTC  GACTATTATT  TCTTGGAATT  TTCTTTTGGA
7151  CCTGGTGCTA  TGGTTGATGG  ATGTATATTG  GATATGTGCG  TTCTGCCAGG
```

FIG. 13 (continued)

```
7201  TGTAAGCACA AAGGTTTAGA CARAMMRARA RCAAGAGCGA GTGAACCTGT
7251  TCTGGTTTTG CAGTGGTTCA GTAAGGCAGA AAGTTGTTAA ACCGTAGTTC
7301  TGAGATGTAT TACCAGTGNC GCCATGCTGT ACTTTTAGGG TGTATAATGC
7351  GGATACAAAT AAACAATTTA GCGGTTCATT AAAGTTTGAA CTCAAATAAC
7401  ATGTTCTTTG TAAGCATATG TACCGTACCT CTACGTGAAA TAAAGTTGTT
7451  GAATTAGCAT TCGAAAAAAA
```

FIG. 13 (continued)

```
   1   MSQLGLAAAA  SKALPLLPNR  QRSSAGTTFS  SSSLSRPLNR  RKSHTRSLRD
  51   GGDGVSDAKK  HSQSVRQGLA  GIIDLPSEAP  SEVDISHGSE  DPRGPTDSYQ
 101   MNGIINETHN  GRHASVSKVV  EFCAALGGKT  PIHSILVANN  GMAAAKFMRS
 151   VRTWANDTFG  SEKAIQLIAM  ATPEDMRINA  EHIRIADQFV  EVPGGTNNNN
 201   YANVQLIVGM  AQKLGVSAVW  PGWGHASENP  ELPDALTAKG  IVFLGPPASS
 251   MNALGDKVGS  ALIAQAAGVP  TLAWSGSHVE  VPLECCLDAI  PEEMYRKACV
 301   TTTEEAVASC  QVVGYPAMIK  ASWGGGGKGI  RKVHNDDEVR  ALFKQVQGEV
 351   PGSPIFVMRL  ASQSRHLEVQ  LLCDQYGNVA  ALHSRDCSVQ  RRHQKIIEEG
 401   PVTVAPRETV  KALEQAARRL  AKAVGYVGAA  TVEYLYSMET  GDYYFLELNP
 451   RLQVEHPVTE  WIAEVNLPAA  QVAVGMGIPL  WQIPEIRRFY  GMDYGGGYDI
 501   WRKTAALATP  FNFDEVDSQW  PKGHCVAVRI  TSEDPDDGFK  PTGGKVKEIS
 551   FKSKPNVWAY  FSVKSGGGIH  EFADSQFGHA  FAYGLSRPAA  ITNMSLALKE
 601   IQIRGEIHSN  VDYTVDLLNA  SDFRENKIHT  GWLDTRIAMR  VQAERPPWYI
 651   SVVGGALYKT  VTTNAATVSE  YVSYLTKGHI  PPKHISLVNS  TVNLNIEGSK
 701   YTIETVRTGH  GSYRLRMNDS  TVEANVQSLC  DGGLLMQLDG  NSHVIYAEEE
 751   AGGTRLQIDG  KTCLLQNDHD  PSKLLAETPC  KLLRFLVADG  AHVDADVPYA
 801   EVEVMKMCMP  LLSPASGVIH  CMMSEGQALQ  AGDLIARLDL  DDPSAVKRAE
 851   PFDGIFPQME  LPVAVSSQVH  KRYAASLNAA  RMVLAGYEHN  INEVVQDLVC
 901   CLDNPELPFL  QWDELMSVLA  TRLPRNLKSE  LEDKYKEYKL  NFYHGKNEDF
 951   PSKLLRDIIE  ENLSYGSEKE  KATNERLVEP  LMNLLKSYEG  GRESHAHFVV
1001   KSLFEEYLTV  EELFSDGIQS  DVIETLRHQH  SKDLQKVVDI  VLSHQGVRNK
1051   AKLVTALMEK  LVYPNPGGYR  DLLVRFSSLN  HKRYYKLALK  ASELLEQTKL
1101   SELRASVARS  LSDLGMHKGE  MSIKDNMEDL  VSAPLPVEDA  LISLFDYSDR
1151   TVQQKVIETY  ISRLYQPHLV  KDSIQMKFKE  SGAITFWEFY  EGHVDTRNGH
1201   GAIIGGKRWG  AMVVLKSLES  ASTAIVAALK  DSAQFNSSEG  NMMHIALLSA
1251   ENESNISGIS  SDDQAQHKME  KLSKILKDTS  VASDLQAAGL  KVISCIVQRD
1301   EARMPMRHTF  LWLDDKSCYE  EEQILRHVEP  PLSTLLELDK  LKVKGYNEMK
1351   YTPSRDRQWH  IYTLRNTENP  KMLHRVFFRT  IVRQPNAGNK  FRSAQISDAE
1401   VGCPEESLSF  TSNSILRSLM  TAIEELELHA  IRTGHSHMYL  CILKEQKLLD
1451   LIPFSGSTIV  DVGQDEATAC  SLLKSMALKI  HELVGARMHH  LSVCQWEVKL
1501   KLDCDGPASG  TWRVVTTNVT  GHTCTIDIYR  EVEEIESQKL  VYHSASSSAG
1551   PLHGVALNNP  YQPLSVIDLK  RCSARNNRTT  YCYDFPLAFE  TALQKSWQSN
1601   GSTVSEGNEN  SKSYVKATEL  VFAEKHGSWG  TPIIPMERPA  GLNDIGMVAW
1651   IMEMSTPEFP  NGRQIIVVAN  DITFRAGSFG  PREDAFFETV  TNLACERKLP
1701   LIYLAANSGA  RIGIADEVKS  CFRVGWSDEG  SPERGFQYIY  LTEEDYARIS
1751   SSVIAHKLEL  DSGEIRWIID  SVVGKEDGLG  VENIHGSAAI  ASAYSRAYEE
```

FIG. 14

```
1801   TFTLTFVTGR TVGIGAYLAR LGIRCIQRLD QPIILTGFSA LNKLLGREVY
1851   SSHMQLGGPK IMATNGVVHL TVPDVLEGVS NILRWLSYVP ANIGGPLPIT
1901   KPLDPPDRPV AYIPENTCDP RAAICGVDDS QGKWLGGMFD KDSFVETFEG
1951   WAKTVVTGRA KLGGIPVGVI AVETQTMMQI IPADPGQLDS HERSVPRAGQ
2001   VWFPDSATKT AQALLDFNRE GLPLFILANW RGFSGGQRDL FEGILQAGST
2051   IVENLRTYNQ PAFVYIPMAG ELRGGAWVVV DSKINPDRIE CYAERTAKGN
2101   VLEPQGLIEI KFRSEELQDC MGRLDPELIN LKAKLQDVNH GNGSLPDIEG
2151   IRKSIEARTK QLLPLYTQIA IRFAELHDTS LRMAAKGVIK KVVDWEESRS
2201   FFYKRLRRRI AEDVLAKEIR QIVGDKFTHQ LAMELIKEWY LASQATTGST
2251   GWDDDDAFVA WKDSPENYKG HIQKLRAQKV SHSLSDLADS SSDLQAFSQG
2301   LSTLLDKMDP SQRAKFVQEV KKVLD
```

FIG. 14 (continued)
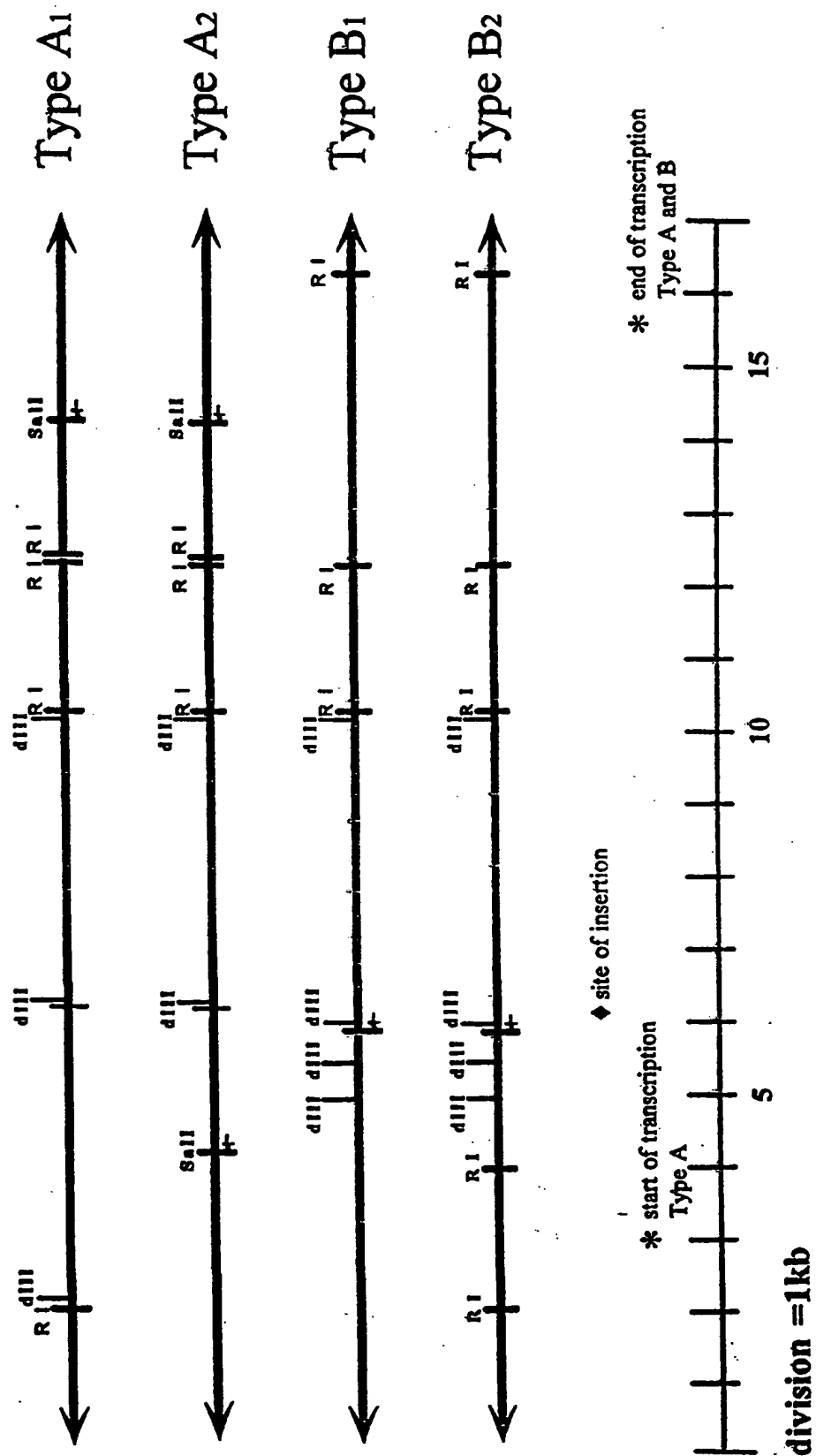
FIG. 15

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```
   1  AAGCTTGGTA TGGATTCgTC AGCGCCAAgC CGGGGTTTTG CATGCGGCCg
  51  ACTGGaArCs GAATTCCgTg AgCCCtGTaC rrCaATGGCA ACCCCAsGGT
 101  TACTggGGTG GCTGAATGGT CTCsGCTTAC GCAATTGTTT GTGGCAgCwG
 151  CGTGGGCTAA ATGTArGTTG TCTCTTGTTG CACTGCArGA TGGATGGGTA
 201  gCCTCTGGGC CGCCTCTGCT ArTGTCTArC GtTTGCTGAC TGTGGTTTAt
 251  TCAgGGATGC CCATGCCCAT GCTAgATTGA tAGGTGCCAT TCTAATGGTA
 301  GGTGGCGGTA AGGTTTATTA AGCTGyAGyA TCAGTAGGTA ACCTCATGAA
 351  TCAGGGTTTA aGCACACCTT TTCCTTTGTG TGGGTGCATA AGGAATGCAC
 401  TTGGCTTCGT TCCCTGATAG TCTTTGsTCA TGTGTCATTC TACCAAGTGG
 451  GTTACTGTAA CATTGCACTC TATGATGGTT GGTGGTtGTG CATCTTTyTG
 501  CTyCCCCTGG yTGTCTAATA CCTGCATGTa ACTGATGACC yyCyTTTATG
 551  TATCATATAG ATTACATCCT TTTGTTGTAC ATCTCAATTC TGAAAAAACA
 601  ATGTTTTGCA TTCTTAGCGc TCTGTGCaCA AgGaAAaGGa gGTTTTACCT
 651  gCAAcTtTTT TTTTCGAGAA AAAACAAACC TTTCTGAAaG gCAGTGATCA
 701  TTTAGtATAA AGAAAATTTG ATTTACTTTC TTCAGAGAGA AtATkCCAAr
 751  CAAACAATTT TCTTACTGTC TGAGCCACGA AATTTGATCT TGATCtTACT
 801  TTCACAAGCC ACATGAAGCC tTATCATCGC TcTGATAAAA AArCCAAaTA
 851  GGTGATTCAT AGAATGAGar AAAGAACCTG TTGCCATTTG GGGaCCTTGT
 901  TGTGTACTCA TTATCCCCCC TGCTCAGGTT GAGGTTTCCT TGCCACTGCC
 951  ACCCCTTGGC CCCTTCTTAT ACAACCATCT CCATTGAAAa AGATTTTGCA
1001  CtACATTTGG GCTTcGTATG aCAAAAaAGG aAAATaAAaC TaAaCAGCAG
1051  AAACATAGTA TAaTTATAGg TAAAAGGTTc TGGCAAGTTT GAGTGGaAGA
1101  GACCTTTGTA TATTTGGACA TATTTCACTA GTAAATAGTT TTCTAAAATc
1151  TTCATGAATG GTGGCCAATA AACTTGATAA GATCTCAACA TGGCAGGTTC
1201  CTTCmAAATG AGAGGAAAAC TGGAAACATC ACAAATATTT TTTAGCGAGT
1251  GGCCTATAAA TTATAATGTT GCTTTCATTT CTTTGATATT CaAAACTTCC
1301  TAAGAGTATT CTGCTAGAGC TCTGATGGTG TCTTTTGCCT CTGTCAGATT
1351  TTCCAGGaGT TTTCTTCCCT TTTtATGGCA CTGTGCGTTT GAGAAGGTCT
1401  TCAATTGTGC TGTCTGGGCC ACGGAACGAC AATGTCACAG CTTGGATTAG
1451  CCGCAGCTGC CTCAAAGGCC TTGCCACTAC TCCCTAATCG CCAGAGAAGT
1501  TCAGCTGGGA CTACATTCTC ATCATCTTCA TTATCGAGGC CCTTAAACAG
1551  AAGGAAAAGC CATACTCGtT CACTCCGTGA TGGCGGAGAT GGgGTATCAG
1601  ATGCCAAAAA GCACAGCCAG TCTGTTCGTC AAGGTACTGt GAATATCTTT
1651  tGaTACAAgc tAAAATTTtG cTACAGAATA TaTAtTtaAA GAGTtCTtTC
1701  TTGGcTGGtG ttGtTTATTT GttT.aACAt sCGAAAgGGC CtctAgttGg
1751  attGgttaGG tggsCTGAAT ACCACTCCTT AAGGTCTTGA GTTTGCTtTT
```

FIG. 16

```
1801  CCCCnCGGAG CGAATTTTAG GCTAGGGTTA CCCCCCCACC CCCACCCGAA
1851  TCTGCACAGy CCGGyCGyGG yCGyCCTCAT ATAGGCTACG ATGTCATTGT
1901  GTATCGGCGG GCCAGGGGTT TAAGAGTTTT CTTGACCTTT GTTAGAAGAT
1951  CTTAATAATA CAATGTCCAA GGGCTGTCTT ACCCTGTAGG TCGAGTTTTT
2001  AGTTGTTTTA ACATGGTAAT GTTTGAAGCC TCATTCTAGG TrCCAATATA
2051  GATATGCTCA CTGCTCAGTT TCAAATGTTT GTCTGCATGT AGGTCTTGCT
2101  GGCATTATCG ACCTCCCAAG TGAGGCACCT TCCGAAGTGG ATATTTCACA
2151  GTAAGGACTA CAATATTTTG CGTACGTTTG TTTTGGAAAA AGAAAATATT
2201  CTCAGCTTAT TTATACTAGC TTCGCTAATA CTGAAATGCT GTCTTAATGT
2251  CCTGGTGCTG TATGCTCAAT CTTTCATAGT AAATGCTGCA AAATATGTGA
2301  TGTAACTGTT GCAACACAGC CAGGGACCTG TTATTTAGAG CATGGTGAAT
2351  GCTCTGGTTC AGTTATATGA TGTAGTTATA GCTCATGTTG AAGAATTAGT
2401  TGCAGTGTTT GCTGGACAAT GGTCACTTAT TATAAATCAT ATCTGCATAC
2451  ACATTTGTGA CTTCTGTTGC TGTAAATGCC CGCATTTTTT GAGAAAAATT
2501  TAAATGCTTG GCCTAAATTG GACATATATG ATAGACAAAG CTGATTTGAA
2551  CTTTGTTTAT TTTTGACATC CATGCATATT GTCAGTGTTG TGAAAACAAT
2601  ACTAATCCTT TTTTTTTGTC TTTTTCCAGT GGATCTGAGG ATCCTAGGGG
2651  GCCAACAGAT TCTTATCAAA TGAATGGGAT TATCAATGAA ACACATAATG
2701  GAAGACATGC CTCAGTGTCC AAGGTTGTTG AATTTTGTGC GGCACTAGGT
2751  GGCAAAACAC CAATTCACAG TATATTAGTG GCCAACAATG GAATGGCAGC
2801  ACCCAAATTT ATGAGGAGTG TCCGGACATG GGCTAATGAT ACTTTTGGAT
2851  CTGAGAAGGC AATTCAACTC ATAGCTATGG CAACTCCGGA AGACATGAGG
2901  ATAAATGCAG AACACATTAG AATTGCTGAC CAATTACGTA GAGGTGCCTG
2951  GTGGAACAAA CAATAATAAC TACGCCAATG TTCAACTCAT AGTGGAGGTT
3001  AGCCTTGCTA ATCTGTTAGT TTACTACTGG TCTGCTGTTT CCTTTATTTG
3051  TTGTATAATG ATTGACATAT TTAAGTAGAG AAATTTATAT TTCTCCTCTG
3101  CTGTTGTGGA AGTCCAATTG TCATCATTAA CTGTGAAATA TTGCAGATGG
3151  CACAAAAACT AGGTGTTTCT GCTGTTTGGC CTGGTTGGGG TCATGCTTCT
3201  GAGAATCCTG AACTGCCAGA TGCATTGACC GCAAAAGGGA TCGTTTTTCT
3251  TGGCCCACCT GsATCATCAA TGAATGCTTT GGGAGATAAG GTCGGCTCAG
3301  CTCTCATTGC TCAAGCAGCC GGGGnCCCAA CTCTTGCTTG GAGTGGATCA
3351  CATGTGAGTC TCACTCTTTG ATTACTATCC GCCTGTCTCA TTGCTCTCTC
3401  TTTCATATTC TAATGACACT AAATTTAGGT TGAAGTTCCA TTAGAGTGCT
3451  GCTTAGACGC GATACCTGAG GAGATGTATA GAAAAGCTT
```

FIG. 16 (continued)

```
   1 GAATTCCGTG AGCCCTGTAC GGCAATGGCA ACCCCAGGGT TACTGGGGTG
  51 GCTGAATGGT CTCGGCTTAC GCAATTGTTT GTGGCAGCTG CGTGGGCTAA
 101 ATGTAGGTTG TCTCTTGTTG CACTGCAGGA TGGATGGGTA GCCTCTGGGC
 151 CGCCTCTGCT AGTGTCTAGC GTTGCTGACT GTGGTTTATT CAGGGATGCC
 201 CATGCCCATG CTAGATTGAT AGGTCATAGG TGCCATTCTA ATGGTAGGTG
 251 GCGGTAAGGT TTATTAAGCT GTCGTATCAG TAGGTAACCT CATGAATCAG
 301 GGTTTAAGCC CACCTTCTCC TTTGTGTGGG TGCATAAGGA ATGCACTTGG
 351 CTTCGTTCCC TGCTAGTCTT TGCTCATGTG TCATTCTACC AAGTGGGTTA
 401 CTGTAACATT GCACTCTATG ATGGTTGGTG GTTGTGCATC TTTTTGCTTC
 451 CCCTGGTTGT CTAATACCTG CATGTAACTG ATGACCTTCT TTTATGTATC
 501 ATATAGATTA CATCTTTTGT TGTACATCTC AATTCTGAAA AACAATGTTT
 551 TGCATTCTTA GCGCTCTGTG CACAAGGAAA AGGAGGTTTT ACCTGCAACT
 601 TTTTTTTTCG AGAAAAAACA AACCTTTCTG AAAGGCAGTG ATCATTTAGT
 651 ATAAAGAAAA TTTGATTTAC TTTCTTCAGA GAGAATATTC CAAACAAACA
 701 ATTTTCTTAC AGTCTGAGCC ACGAAATTTG ATCTTGATCT TACTTTCACA
 751 AGCCACATGA AGCCTTATCA TCGCTCTGAT AAAAAAACCA AATAGGTGAT
 801 TCATAGAATG AGAAAAAGAA CCTGTTGCCA TTTGGGGACC TTGTTGTGTA
 851 CTCATTATCC CCCCTGCTCA GGTTGAGGTT TCCTTGCCAC TGCCACCCCT
 901 TGGCCCCTTC TTATACAACC ATCTCCATTG AAAAAGATTT TGCACTACAT
 951 TTGGGCTTCG TATAACAAAA AAGGAAAATA AAACTAAACA GCAGAAACAT
1001 AGTATAATTA TAGGTAAAAG GTTNTGGCAA GTTTGAGTGG TAGAGACCTT
1051 TGTATATTTG GACATATTTC ACTAGTAAAT AGTTTTCTAA AATGTTCATG
1101 AATGGTGGCC AATAAACTTG ATAAGATCTC AACATGGCAG GTTCCTTCAA
1151 AATGAGAGGA AAACTGGAAA CATCACAAAT ATTTTTTAGC GAGTGGCCTA
1201 TAAATTATAA TGTTGCTTTC ATTTCTTTGA TATTCAAAAC TTCCTAAGAG
1251 TATTCTGCTA GAGCTCTGAT GGTGTCTTTT GCCTCTGTCA GATTTTCCAG
1301 GAGTTTTCTT CCCTTTTTAT GGCACTGT
```

FIG. 17

```
   1  AGCATCCCTT  GGGATTGTGA  TnACTCACAT  AAATTCTTGC  GAAnTGTTGA
  51  CATTCTAGTG  ATTTGAGTTC  CGTTCTAGTG  TGCTAGTCAn  TTGAGCTCAA
 101  GTCTTGGTTT  TATGTGTGCG  TATTCACTGT  GATCTTTGTG  TCGTGTGTGA
 151  GTTGTTGATC  CTTCCCTTGC  TCCGTGATTC  TTTGTGAAAT  CTTTTGAAAG
 201  GGCGAGAGGC  TCCAAGCTGT  GGAGATTCCT  CGCAAGTGGG  ATTAAGAAAA
 251  GCAAAGCAAC  ACCGTGGTAT  TCAAGTTGGT  CTTTGGACCG  CTTGAGAGGG
 301  GTTGATTGCA  ACCCTCGTCC  GTTGGGACGC  CACAACGTGG  AGTAGGCAAG
 351  CGTTGGTCTT  GGCCGAACCA  CGGGATAACC  ACCGTGCCAT  CTCTGTGATT
 401  GATATCTCTT  GGTTATTGTG  TTGTGTTGAG  ATCCTTCTCT  AGCCACTTGG
 451  CAAATTACTG  TGCTAACAAT  TAATCAAGTT  TTGTGGCTTA  AGATTTTGAA
 501  GTATTACAGG  ATCTGCATCA  TGGTCTGTGT  CTCCACAGCT  ATGACAGCCA
 551  CAGGAATTCA  TGTGTTCCTT  GGAGCCACTC  TTGGATGACC  TAAAGGAATT
 601  ATTTCTAACC  GGCTTGTACA  CATATGATGC  ATCAAGAGAT  GAGTGTTTTA
 651  CTATGCGAGG  GGCCATGCTT  ATGACCATAA  GTAATCTTCC  TGGTTTAGAA
 701  ATGCTTGCTT  CTCATATGGT  TCATGGGAAA  TTCGCATGCC  TCCTTGTGGT
 751  GAAAATGTCT  GGACAAAACA  GCTGAAGAAT  GGTCGTAAAT  CTTGTTTTAT
 801  GGGAAATCGC  CAATATATTG  ATCTTGATCA  TTCTTATTGC  TTGGATGCAG
 851  ACTCCGTTTG  ATGGAACGAT  AGACTTCGAA  CAAAACCTAA  AACCTATTAT
 901  GATCGTCCAA  TTTTGGATGA  AATCATCACA  CTTGGTGATT  TCAAGAACTC
 951  AAAAAyTTAC  AGTTAATTGG  ATATAGGAGG  GnGCAAAAAC  ACAGTAAGTT
1001  GGACATTCCA  TAAGGGGATT  TATTTTAGTT  GACAATAAAG  TAGATGGGCA
1051  TCATCCTGAG  TTTnGTTTGG  CATCGTGTCG  TAGATTGAAA  CTGTAAGGAT
1101  GGACATGGTA  GnTAACAGGT  TGAGATGAAT  GATTCAACAG  TTGAAGCGAA
1151  TGTACAATCT  TTATGTGATG  GTGGCTnTTA  ATGCAGGTAA  CTAGTTTTTT
1201  TTTATGCTTT  ATTATTAATT  AGTTGGATAA  ATGGTTTnGA  TTTnTGATTG
1251  TTAAAnTGCA  ATGGCTCCAG  TTGGATGGnA  ACAGCCAnGT  AATTTATGCA
1301  GAAGnAGnAG  nTGGTGGTAC  ACGGnTTCAG  ATTGATGGAA  AGAnATGTTT
1351  ATTGCAGGTA  AATAnTCCCT  TnTTCCTTTA  TATTTTTGTT  GTnTGATTGT
1401  ATAAnTTTGn  TAGATTATTT  GTATAATTTA  TTATTGCATT  TCACCCCACT
1451  AAnTTATTTT  TAAAAGATGG  GTTTTGTTGT  TTGnTTCAGC  nGGCGACATC
1501  ACATAAGnAA  ATTGTGATTA  ATTTTTGTTT  TTTTGCAGnA  TGACCATGAT
1551  CCATCAAAGT  TATTA
```

FIG. 18A

```
   1  ACATAAGCTG GGTTAGTAGT GGTGAATTAG TGGATTATTG ATCTGTTGTG
  51  GGCAAGGAGn TGGCTTGGTG TCGAGAAAAA ACATGGAaGT GTGTATTGCC
 101  AGTGcTTaTT yTrGGGATAT GAGGGAATTw AmATTAcATT TGTGAcTGGG
 151  CGGACTGTAG GATAGGAGTT ATCTTGyTcG ATTGGTATAC GGTGCATACA
 201  GskyTTGACC AGCTATTATT TTAACAGGgT TTTCTGCCCT GAACAAGTCC
 251  TTGGGCGGGA AGTGTACAGc TCCCACATGC AGCTTGGTGG TCCTAAGATC
 301  ATGGCGACCA ATGGTGTTGT CCACCTCACT GTTCCAGATG ACCTTGAAGG
 351  TGTTTCCAAT ATATTGAGGT GGCTCAGCTA TGTTCCTGCA AACATTGGTG
 401  GACCTcTTCC TATTACCAAA CCTCTGGACC CTCCAGACAG ACCTGTTGCT
 451  TACATCCCTG AGAACACATG CGATGCACGT GCAGCTATCT GTGGTGTAGA
 501  TGACAGCCAA GGGAAATGGT TGGGTGGTAT GTTTGACAAA GACAGCTTTG
 551  TGGAGACATT TGAAGGATGG GCAAAAACAG TGGTTACTGG CAGAGCAAAG
 601  CTTGGAGGAA TTCCATGCAT CTTAATAAAC ACAGTTGGCC CTTAAAGCAA
 651  GTGAACTTCT TGAACAAACC AAACTAAGTG AACTCCGTGC AAGCGTTGCA
 701  AGAAGCCTTT CGGATCTGGG GATGCATAAG GGAGAAATGA GTATTAAGGA
 751  TAACATGGAA GATTTAGTCT CTGCCCCATT ACCTGTTGAA GATGCTCTGA
 801  TTTCTTTGTT TGATTACAGT GATCGAACTG TTCAGCAGAA AGTGATTGAG
 851  ACATACATAT CACGATTGTA CCAGGTATTA TATCAACTAA CTTAATGTCT
 901  TCCATAGTCT CACTAAGCAT ATCTGATATG tTTAGATACC CTACATGGAA
 951  TGCTCATCTT TTCATTTGAC ACAAGAAAC ATTGAGAAAT GAGATGCTGA
1001  CGATTGGCTG AAATTAACTG GGTnTGAGAA ATTGTGATCT CCCAACTTGT
1051  TAATGCACAA TGTTCTGGCT AACTTGCCAA TATTTTTTCA GCCTCATCTT
1101  GTTnAGGATA GCAnCCAAAT GAAATCCAAG GATCTGGTGC TATTACTTTT
1151  TGGGAATTTA TGAAGGGC
```

FIG. 18B

```
  1  CTCCCAATAT  TGTCATGAGG  CTTGCATCCC  AGGTTAGTTT  TTTTTGCTTT
 51  CTGAAATTTA  TATTCCATAC  CTTTTCAGCT  TTAGTTATCC  TTGTATTTTC
101  TGGAAGCTTC  ATCTGATGCA  TTATTGACAA  ATGCACTAAT  GGTCATCATA
151  TTTGGAGATT  AACATATTTA  TCTTAATTGA  TGGGAACTCT  TGAAAATGAC
201  AATGGTTGAG  CAGATAATTA  ACAGTTTTTT  AATAAAAAAA  CATGCATTTC
251  TAGGAGTTGG  ACTAAGCTTT  TCTTAGTATG  AAGTGCCATG  TTTTACATGG
301  TCCATTTGTG  TCAATTTACA  GTCGGTATCA  TGGAAAGGTT  GTCATAATGG
351  CTGGAGANAA  ACAACACATC  TTGTTTCTCA  ACACTTGTGG  GAGAAGANGT
401  TTTACCTTTT  TTCCTAAAAT  TACTTTTTGT  ACTAAATTGT  ATAATTTTTC
451  CAATATTCTC  CATGATTATT  GAACTCTGCT  GTGTTCAAAC  AGCCAAAACA
501  TGTTTCCATA  CTTTACACCT  TTATTTTTTA  GATGGAACCT  GGAATTGTGC
551  TCTGTTATCT  GTATCATGCA  TATATTGATC  TTAAACCTAT  CTCTATTGTA
601  GAATCCGCAC  TTGAATTCAG  TTGCTTGTGA  TCAATATG
```

FIG. 18C

```
  1  GGTAACCACC ACACCCGCGG CGCTTAATGG CCGTACAGGG NGGTCCCATT
 51  CGCCATTCAG GTGCGCAACT GTTGGGAAGG GCGATCGGTG CGGGCTTCTT
101  CGNTATTACG CCAGCTGGCG AAAGGGGGAT GTGCTGCAAG GCGATTAAGT
151  TGGGTAACGC CAGGGTTTTC CCAGTCACGA CGTTGTAAAA CGACGGCCAG
201  TGAGCGCGCG TAATACGACT CACTATAGGG CGAATTGGGT ACCGGGCCCC
251  CCCTCGAGGT CGACCTGCAG GTCAACGGAT CCTAGGGGGC CAACAGATTC
301  TTATCAAATG AATGGGATTA TCAATGAAAC ACATAATGGA AGACATGCCT
351  CAGTGTCCAA GGTTGTTGAA TTTTGTGCGG CACTAGGTGG CAAAACACCA
401  ATTCACAGTA TATTAGTGGC CAACAATGGA ATGGCAGCAG CAAAATTTAT
451  GAGGAGTGTC CGGACATGGG CTAATGATAC TTTTGGATCT GAGAAGGCAA
501  TTCAACTCAT AGCTATGGCA ACTCCGGAAG ACATGAGGTA AATGCAGAAC
551  ACATTAGA
```

FIG. 18D

```
   1   GAATAATCTG CCTGCAGCTC AAGTTGCTGT TGGAATGGGC ATACCTCTTT
  51   GGCAGATTCC AGGTAATTAC CAATTTACCA ACTTATTTAG TTCCTTATTG
 101   TTTTATTCTC TAATTTTCTA CTTATGTAgA AATCAGACGT TTCTATGGAA
 151   TGGACTATGG AGGAGGGTAT GACATTTGGA GGAAAACAGC AGCTCTTGCT
 201   ACACCATTTA ATTTTGATGA AGTAgATTcT CAATGGCCAA AGGGCCATTG
 251   TGTAgCAGTT AGAATTACTA GTGAGGACCC AGATGATGGT TTCAAACcTA
 301   CTGGTGGGAA AGTGAAGGTA AGTTTTCTAG ATGACATGTA TTATATATCG
 351   TTCAAAgAgA TTAAGTTTGG TTAAATGAcT AGGTCTTGAT TTTTTATCTT
 401   TCAGGAGATA AGTTTTAAAA GCAAGCCTAA TGTTTGGGCC TaCTTCTCAG
 451   TAAAGGTaAC TTGTTAACTT TAGTACGCTG TCACATTATt ctTCsTTGTG
 501   .AAAATAAtT TGAACGGTtC TCTTTGTATT TTaACCAtCC AtCgTCTCAT
 551   TTAsCAgAgC ACACAAATAT TtGCACTGAC GCCCcTcCCC tTATCtGCtT
 601   TCAgTCTGGT GGAgGCATtC AtGAATTtGC TGATTCTCAG TTCGGTATGT
 651   GTAAACCAAG AGTATTCTTT GTAATTTATA TTGGTCCTCA ATTTTGAAAT
 701   ATTGcTCTTT CCGTTACAGG ACAwGTTTTT GCATATGGGC TCTCTAGATC
 751   AGCAGCAATA ACAAACATGA CTCTTGCATT AAAAnAGATT CAAATTCGTG
 801   GAGAAATTCA TTCAAATGTT TGATTACACA GTTGATCTCT TAAATGTTAA
 851   GAAATATTAA CCACCTTTTA AATCACATTT TCCATTATGT TTGATTGCAT
 901   ATCATTAATT TTGATTTTCT ATTATGGCTA AACCTGTGGT GCTATTTTCC
 951   TATTATCCCA GGCTTCCGAC TTTAGA
```

FIG. 18E

```
                                                    GGATCC TAgGGGGCCA
1401  ACAGATTCTT ATCAAATGrA aTGGGATTAT CAATGAAACA CATAATGGAA
1451  GACATGCCTC AGTGTCCAAG GTTGTTGAAT TTTGTGCGGC ACTAGGTGGC
1501  AAAACACCAA TTCACAGTAT ATTAGTGGCC AACAATGGAA TGGCAGCAgC
1551  aAAATTTATG AggAGTGTCC GGACATGGGC TAATGATACT TTTGGATCTG
1601  AGAAgGCAAT TCAACTCATA GCTATgGCAA CTCCGGAAGA CATGAGGATA
1651  AATGCAGAAC ACATTAGAAT TGCTGACCAA TTaCGTAgAr gTGcctgGTG
1701  gaacaaACAA tA.ATAActA cgCCaATGTT CAAcTcATAg TGGAaGTTAG
1751  CCTTGcTAAT CTGTTAgTTT ACTACTGgTC tGCtGTTtCC TTtATTtGtt
1801  GTaTAATGAT tGACaTATTt AagTAgAgAA atTTATAtTT CtCctCtgCt
1851  GTTGTGgAag TCCAatTGTC acCATtAACt GTgAAaTATt gCAgATgGca
1901  CaAAAACtAG gTgtTTCTgC TGTTTGGCCT gGTTGGGGTC ATGCTTCTGA
1951  GAATCcTGAA CTGCCAGATG CATTGACCGC AAAAGGGATC GTTTTTCTTG
2001  GCCCACCTGc ATCATCAATG AATGCTTTGG GAGATAAGGT CGGCTCAGCT
2051  CTCATTGCTC AAGCAGGCGG GGtCCCAACT CTTGCTTGGA GTGGATCACA
2101  TGTGAGTCTC ACTCTTTGAT TACTATCCGC CTGTCTCATT GCTCTCTCTT
2151  TCATATTCTA ATGACACTAA ATTTAGGTTG AAGTTCCATT AGAGTGCTGC
2201  TTAGACGCGA TACCTGAGGA GATGTATAGA AAAGCTT
```

FIG. 18F

```
  1  AATTCCTGTG GGTGTTATAG CTGTGGAGAC ACAGACCATG ATGCAGCTCA
 51  TCCCTGCTGA TCCAGGTCAA CTTGATTCCC ATGAGCGATG TGTTCCTCGG
101  GCTGGACAAG TGTGGTTCCC AGATNCTGCA ACCAAGACAG CTCAGGCATT
151  ATTAGACTTC AACCGTGAAG GATTGCCTCT GTTCATCCTG GCTAACTGGA
201  GAGGCTtCTC TGGGGgACAG AGAGATCTCT T
```

FIG. 19A

```
  1  AATTCATGCA TCTTAATAAA CACAGTTGGC CCTTAAAGCA AGTGAACTTC
 51  TTGAACAAAC CAAACTAAGT GAACTCTGTT CCAGCATTGC AAGAAGCCTT
101  TCAGATCTGG GGATGCATAA GGGAGAAATG ACTATTAAGG ATAGCATGGA
151  AGATTTAGTC TCTGNCCCAT TGCCTGTTGA AGATGCTCTT ATTTCTTTGT
201  TTGATTA
```

FIG. 19B

```
  1  ATAGACCTGT CGCATACATC CCTGAGAACA CATGCGATCC GCGTGCAGCC
 51  ATCCGTGGnG TAGATGACAG CCAAGGGAAA TGGTTGGGTG GTATGTTTGA
101  CAAAGACAGC TTTGTGGAGA CATTTGAAGG ATGGGCAAAA ACAGTGGTTA
151  CTGGTAGAGC AAAGCTTGGA GGAAGGAATT
```

FIG. 19C
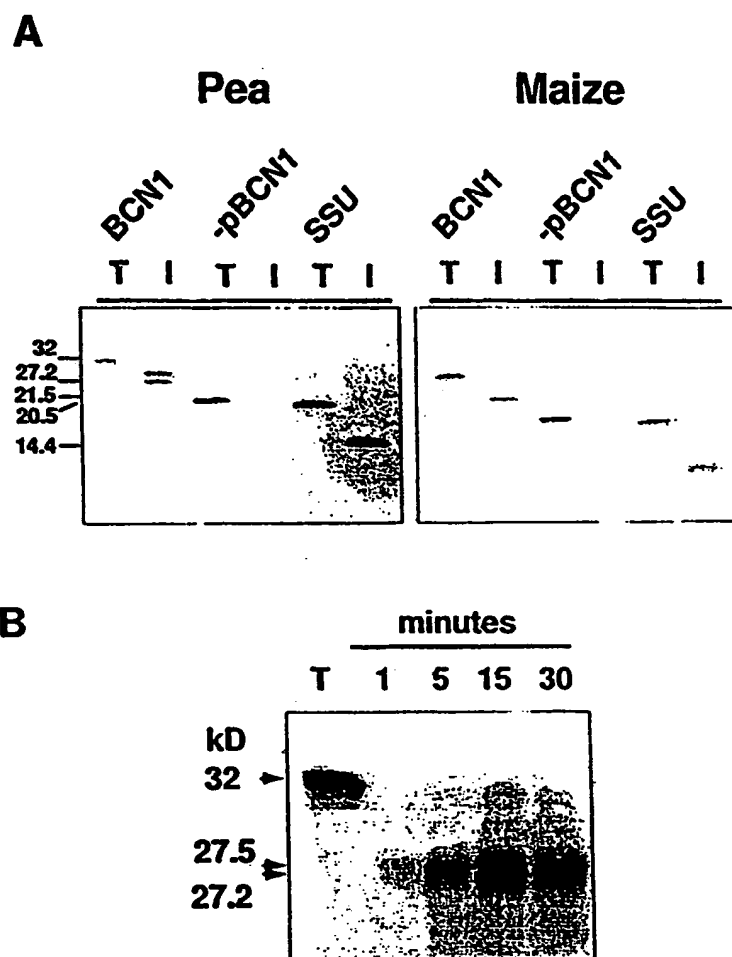
FIG.20

1 ACAAAATAGT AAAGACCTCC AGAAGGTTGT AGACATTGTT TTGTCTCACC

51 AGGGTGTGAG AAGCAAAACC AAGCTgAtAC TCaCGCtCAT gGAgAAACTG

101 GTCtATCCaA ACCCTgcTGC CtATAGGGaC CAGcTgATTC GCtTTTcTTC

151 CCTCAACCAT aAAAGaTaTT aCAAGcTGGC CCTTaAAgcT AgTgAACTTc

201 TcGaACaAAC CaAGcTCAgT gaACtCCGca CAAGCATTGT AaGGAaCcTT

251 tcAGCgCTGg AgATGTTTAC TGAAGAAAGG GCAGGTTTCT CGTTGCAAGA

301 GAAGAAATTG GCCATTAATG AGAGCATGGG AGATTTAGTC AGTGCCCCAC

351 TGCCAGTTGA AGATGCACTT ATATCTTTGT TTGATTGTAT TGATcAAACT

401 CTTCAGCAGA GAGTGATCGA GACGTACATA TCTCGATTAT ACCAGCCTcA

451 ACTTGTGAAG GATAGTATCC AGCTGAAATA TCAGGATTCT GCTGtTATCG

501 CTTTATGGGA ATTCAACGAA GGGCATCCTG ATAATAGATT GGGTGCTATG

551 GTTATCCTGA AGTCTCTAGA ATCTGTGTCA ACAGCAATTG GAGCTGCTCT

601 AAAGGATAGA TCACATTATG CAAGCTCTGC GGGTAACACA GTGCATATTG

651 TTTTGTTGGA TGCTGATACT CAGACGAATA CAACTGAAGA TAGTGGTGAT

701 AACGACCAAG CTCAAGACAG GATGGACAAA CTCTCTGTAA TACTGAAACA

751 AGATATTGTC ACGGCTGATC TATGTGCTGC TGGTGTCAAG GTTATTAGTT

801 GCATTGtTCA AAGAGATGGA GCACTCATGC CTATgCGCCG TACCTTCCTC

851 TTGTCTGAAG AAAAGCTTTG TTATGAGGAA GAGCCGATTC TTCGGCATGT

901 GGAGCCTCCA CTTTCTGCAC TTCTTGAGTT GGATAAATTG AAAGTGAAAG

951 GATaCAATGA GATGAAGTAT ACACCGTCAC GTGATCGTCA GTGGCATATA

1001 TACACACTTA GAAATACTGA AAACCCCAAA ATGTTGCACA GGGTATTTTT

1051 CCGAACTCTT GTCAGACAGC CGAGTGCTGG CAACAGGTTT ATGTCAGGCC

1101 ATATCAATGA TGTTGAAGTA GGACATGCTG AGGAATCTCT TTCATTTACA

1151 TCAAGCAGCA TATTAAGATC TTTGATGACT GCTATAGAAG AATTGGAGCT

1201 TCACGCGATT AGGACTGGTC ATTCTCATAT GTACTTGTGC ATATTGAAAG

1251 AGCAAAAGCT TCTTGACCTT GtTCCTGTTT CAgGGAACAC AGTTGTGGAT

1301 GTTGGCCAAG ATGAAGCTAC TGCGTGCTCT CTTATGAAAG AAATGGCTCT

1351 AAAGATACAT GAACTTGTTG GTGCAAGAAT GCATCATCTT TCTGtATGCC

1401 AGTGGGAAGT gAAACTTAAG TTGGACAGTG ATGGACCTGC CAGTGGTAGC

1451 TGGAGAGTTG TAACAACCAA TGTCACTAGT CACACCTGCA CTGTGGATAT

1501 CTACCGGGAG GTCGAAGATa CAGATTCACA GAAGCTAGTA TACCACTATG

Figure 21

```
1551 CTGCATCGTC ACCTGGTCCT TTGCATGGTG TCACATTGAG TAATTCATAC

1601 CAGCCTTTGA GCGTTATTGA TCTAAAACGA TGCTCTGCCA GGAACAACAG

1651 AACTACATAC TGCTACGATT TTCCATTGGC ATTTGAAACT GCAGTGCGGA

1701 AGTCATGGGC TAACGTTTCt AGTgAAAaCA aCCAATGTtA TGTTAAagCA

1751 aCAgAGcTGG TGTTtGCTgA CAAGAATGGG TCGTGGGGCA CTCCTATAGT

1801 TCCTATGCAG CGTGCTGCTG GGCTCAATGA CATCGGTATG GtTGCgTGGA

1851 TCtTGGaCAT GTCCaCTCCT GAATTTCCCA GTGGCAGACA GATTATTGTT

1901 ATCGCAAATG ATATTACTTT CAGAGCTGGA TCATTTGGCC CAAGGGAAGA

1951 TGCATTTTTT GAAGCTGTTA CCAACCTGGC TTGTGAGAGA AAGCTTCCTC

2001 TTATCTACAT GGCAGCAAAC TCTGGTGCTC GGATTGGCAT TGCTGATGAA

2051 GTAAAATCTT GCTTCCGTGT TGAATGGGTT GATCCTGCCa ACGCTGAACG

2101 TGGATTTAAG tACATTTATC TGAATgAAGA AGaCTATGGT CGTAtTAgCT

2151 cTTCTGtTAt AGCGCACAAg aCaCAgcTag ATagtGGCgA AAtAaGgtgG

2201 GtTAtCGATt CTGTTGtGGg CAAGGAGGAT GGaCTAGGTG TGGAGAACAT

2251 ACATGGAAgT GCTGcTAtTG CCAGTGCTTA TtCTAGGgCA tACgAgGAGA

2301 CaTTtACaCT TACATTtGTG AGTGGtCgAA CGGTTgGAAT AGgAgGCcTAT

2351 cTTGCTcGGc TTGGcAtaCG GtGCAtACAG cGTATTGaCC AGcCCATTAT

2401 CTtAAcTgGG TTTTCTGCCc TGaACAAGCT TCTCGGGCGG gAAGTGTACA

2451 GCTCCCACAT GCAGTTGGGT GGTCCCAAAA TCATGGCTAC AAAtGGTGtT

2501 GTCCATCTGA CTGTTCCAGA TGACCTTgAA GGTGtTtCtA ATATAtTGAG

2551 GTGGCTCAgT TATGTTGCTG CTAACATTGG TGGACCTCTT cCTATTaCaA

2601 AATCTTTAGA CCCCAAtaGAC AGACCTGtTG cATACATcCC TGaGAATaCa

2651 TGtGAtCCTc GTGCAGCCAT CAGTGGCATT GATGACAGCC AAGGGAAATG

2701 GTTGGGTGGT ATGTTTGACA AAGACAGTTT TGTGGAGACA TTTGAAGGAT

2751 GGGCAAAGAC AGTAGTTACT GGCAGAGCAA AACTTGGAGG GATTCCTGTG

2801 GGTGTTATAG CTGTGGAGAC ACAGACCATG ATGCAGCTCG TCCCTGCAGA

2851 TCCAGGCCAG CCTGATTCCC ATGAGCGGTC TGTTCCTCGT GCGGGGCAAG

2901 TCTGGTTTCC AGATTCAGCT ACCAAGACAG CGCACGCAAT GTTGGACTTC

2951 AACCGTgAAG GATTACCTCT GTTCATCCTt GCTAACtGGA GAGGCTTCTC

3001 TGGTGGGCAA AGAGACCTTT TTGAAGGAAT T
```

```
   1 QNSKDLQKVV DIVLSHQGVR SKTKLILTLM EKLVYPNPAA YRDQLIRFSS

  51 LNHKRYYKLA LKASELLEQT KLSELRTSIV RNLSALEMFT EERAGFSLQE

 101 KKLAINESMG DLVSAPLPVE DALISLFDCI DQTLQQRVIE TYISRLYQPQ

 151 LVKDSIQLKY QDSAVIALWE FNEGHPDNRL GAMVILKSLE SVSTAIGAAL

 201 KDRSHYASSA GNTVHIVLLD ADTQTNTTED SGDNDQAQDR MDKLSVILKQ

 251 DIVTADLCAA GVKVISCIVQ RDGALMPMRR TFLLSEEKLC YEEEPILRHV

 301 EPPLSALLEL DKLKVKGYNE MKYTPSRDRQ WHIYTLRNTE NPKMLHRVFF

 351 RTLVRQPSAG NRFMSGHIND VEVGHAEESL SFTSSSILRS LMTAIEELEL

 401 HAIRTGHSHM YLCILKEQKL LDLVPVSGNT VVDVGQDEAT ACSLMKEMAL

 451 KIHELVGARM HHLSVCQWEV KLKLDSDGPA SGSWRVVTTN VTSHTCTVDI

 501 YREVEDTDSQ KLVYHYAASS PGPLHGVTLS NSYQPLSVID LKRCSARNNR

 551 TTYCYDFPLA FETAVRKSWA NVSSENNQCY VKATELVFAD KNGSWGTPIV

 601 PMQRAAGLND IGMVAWILDM STPEFPSGRQ IIVIANDITF RAGSFGPRED

 651 AFFEAVTNLA CERKLPLIYM AANSGARIGI ADEVKSCFRV EWVDPANPER

 701 GFKYIYLNEE DYGRISSSVI AHKTQLDSGE IRWVIDSVVG KEDGLGVENI

 751 HGSAAIASAY SRAYEETFTL TFVSGRTVGI GAYLARLGIR CIQRIDQPII

 801 LTGFSALNKL LGREVYSSHM QLGGPKIMAT NGVVHLTVPD DLEGVSNILR

 851 WLSYVPANIG GPLPITKSLD PIDRPVAYIP ENTCDPRAAI SGIDDSQGKW

 901 LGGMFDKDSF VETFEGWAKT VVTGRAKLGG IPVGVIAVET QTMMQLVPAD

 951 PGQPDSHERS VPRAGQVWFP DSATKTAHAM LDFNREGLPL FILANWRGFS

1001 GGQRDLFEGI
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Figure 22

FIGURE 33

Figure 24

A

B
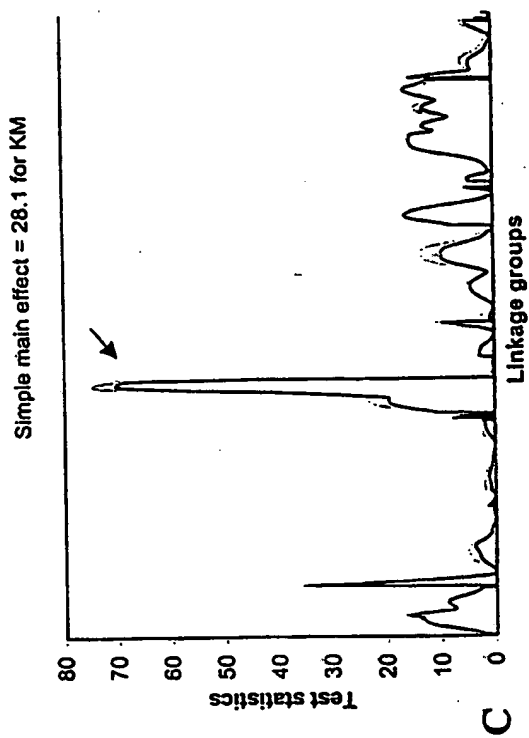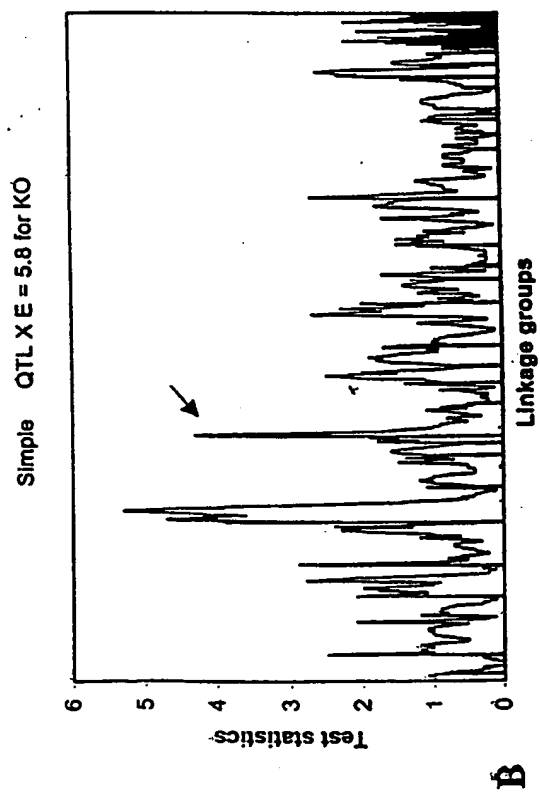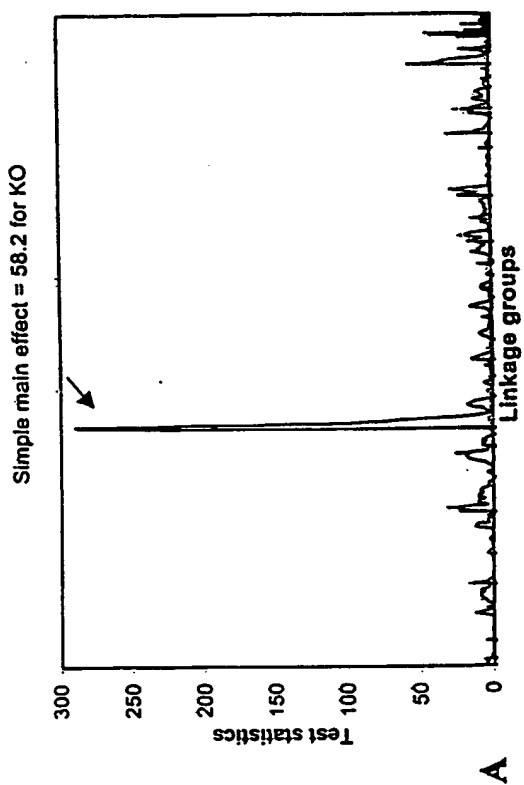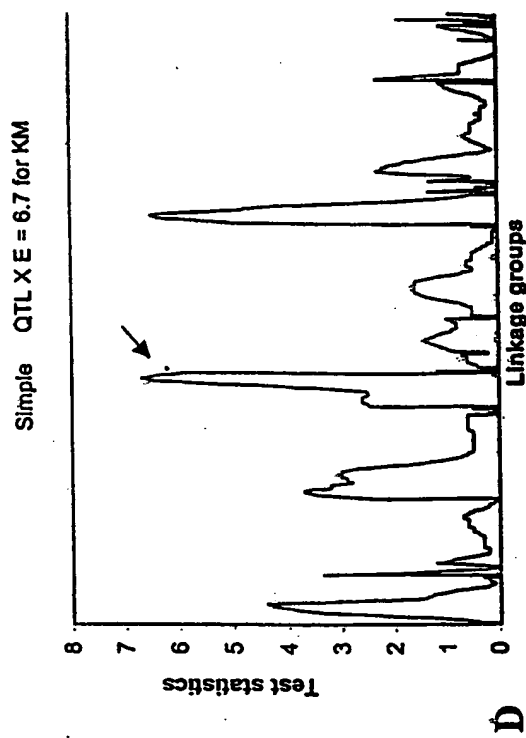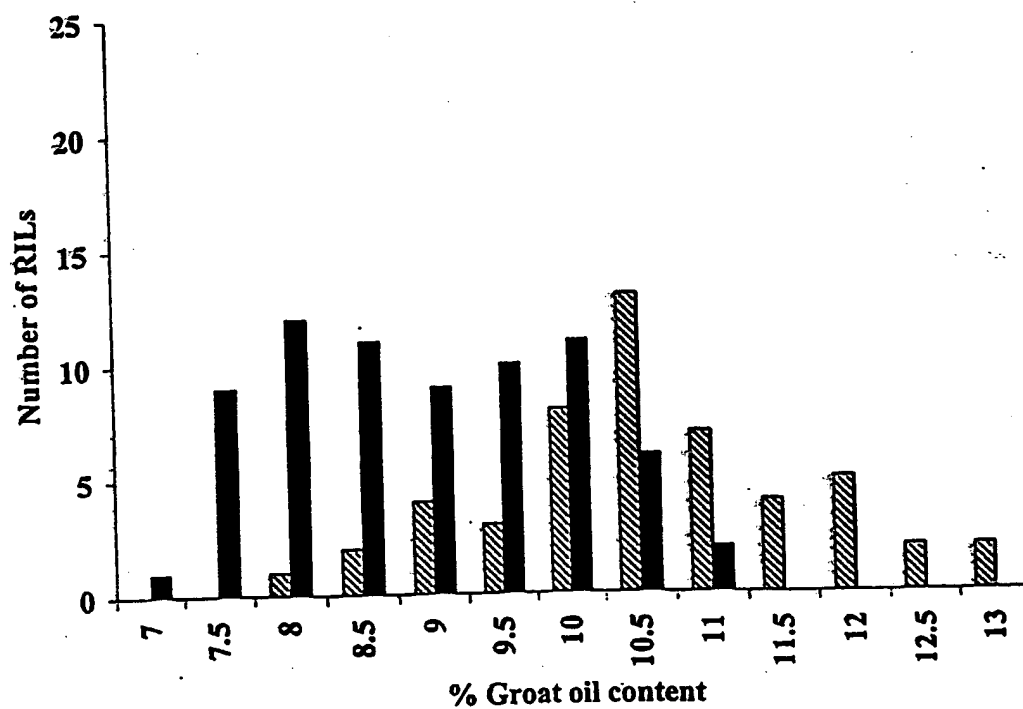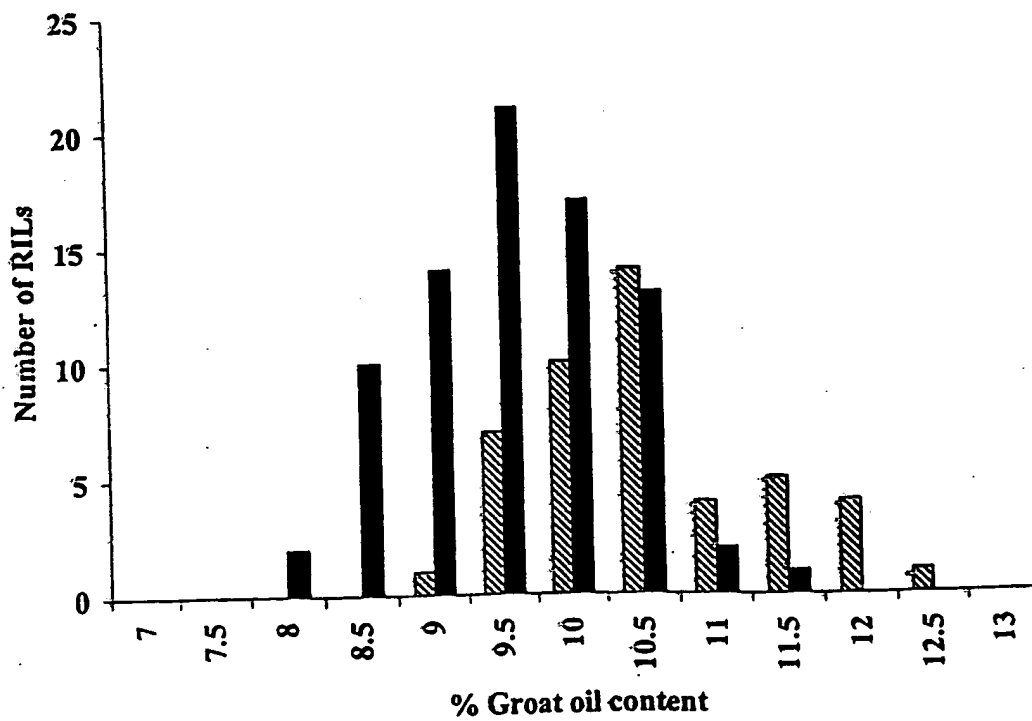
Figure 25.
Phylogram of ACCases based on
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protein distances for 121
ungapped amino acids.

Chloroplast transit peptides
on completely sequenced plant
ACCases are noted as present
(+) or absent (-).

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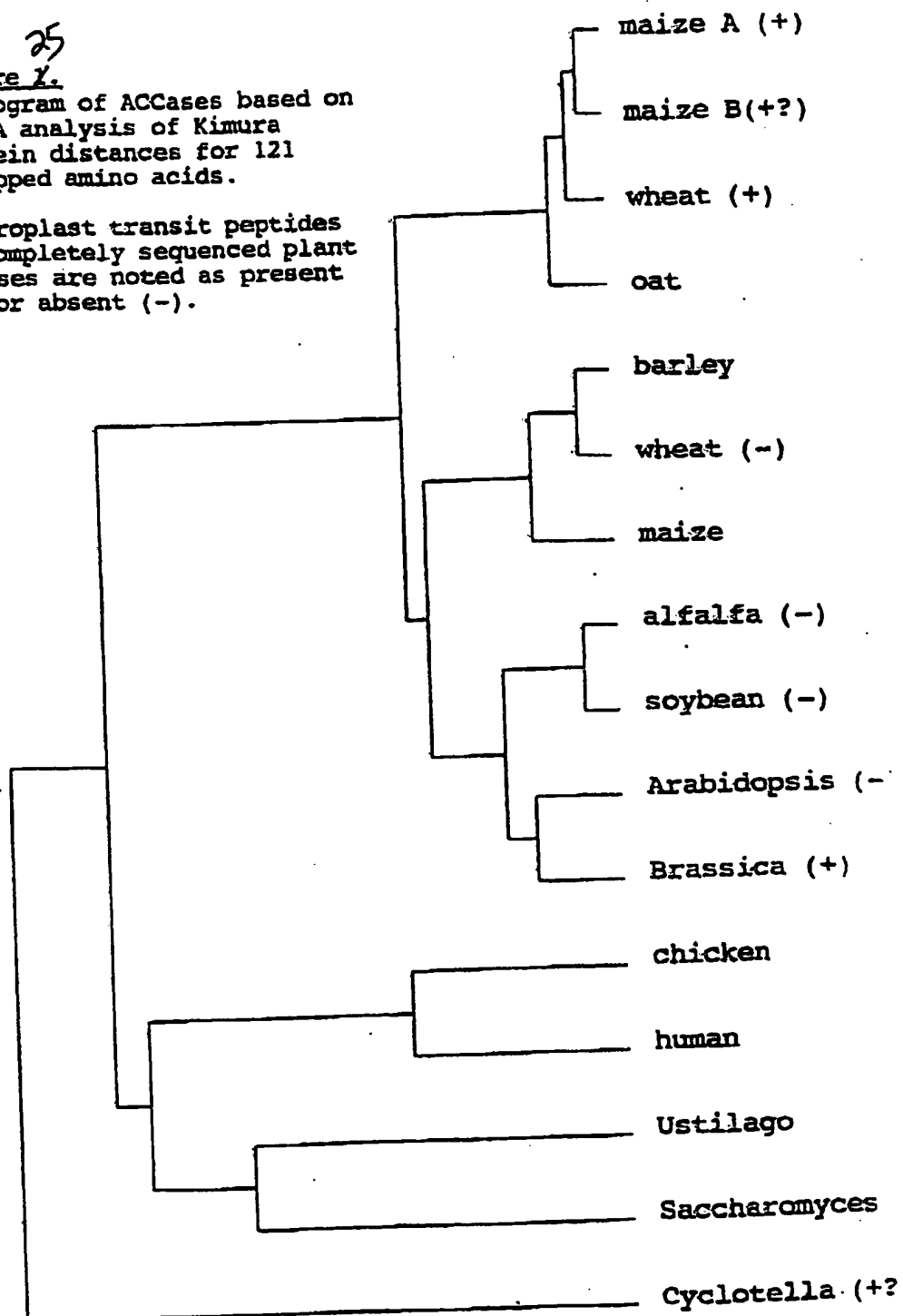
SEQUENCE LISTING

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<223> "n" represents "a" or "g" or "c" or "t"

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gcnaaggtag gatgtcccga agaatctctt tcatttacat caaatagcat cttaagatca   360
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2

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gttactggtc acacctgcac cattgatata taccgagaag tggaggaaat agaatcacag    720
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```

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        <211> 258
        <212> DNA
        <213> Zea mays


        <400> 2
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gacagccaag ggaaatggtt gggtggtatg tttgacaaag acagctttgt ggagacattt    180
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        <210> 3
        <211> 4
```

3

```
        <212> PRT
        <213> Zea mays


        <400> 3
5 Val Met Lys Met
    1


        <210> 4
        <211> 26
10      <212> DNA
        <213> Zea mays


        <400> 4
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15

        <210> 5
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        <212> DNA
        <213> Zea mays
20

        <220>

        <221> unsure
        <222> (7319)...(7319)
        <223> "n" represents "a" or "g" or "c" or "t"
25
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    ttctcatcat cttcattatc gaggccctta aacagaagga aaagccatac tcgttcactc       180
30 cgtgatggcg gagatggggt atcagatgcc aaaaagcaca gccagtctgt tcgtcaaggt       240
    cttgctggca ttatcgacct cccaagtgag gcaccttccg aagtggatat ttcacatgga       300
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    aaaacaccaa ttcacagtat attagtggcc aacaatggaa tggcagcagc aaaatttatg       480
35 aggagtgtcc ggacatgggc taatgatact tttggatctg agaaggcaat tcaactcata       540
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    gggatggcac aaaaaactagg tgtttctgct gtttggcctg gttggggtca tgcttctgag       720
    aatcctgaac tgccagatgc attgaccgca aaagggatcg tttttcttgg cccacctgca       780
40 tcatcaatga atgctttggg agataaggtc ggctcagctc tcattgctca agcagccggg       840
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-4

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     acaccattta attttgatga agtagattct caatggccaa agggccattg tgtagcagtt   1620
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     aattctacag ttaatttgaa tatagaaggg agcaaataca caattgaaac tgtaaggact   2160
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     agaagccttt cggatctggg gatgcataag ggagaaatga gtattaagga taacatggaa   3420

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  5  gatcgaactg ttcagcagaa agtgattgag acatacatat cacgattgta ccagcctcat   3540

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6

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    1             5                 10                  15
    Leu Pro Asn Arg Gln Arg Ser Ser Ala Gly Thr Thr Phe Ser Ser Ser
                20                  25                  30
 5  Ser Leu Ser Arg Pro Leu Asn Arg Arg Lys Ser His Thr Arg Ser Leu
                35                  40                  45
    Arg Asp Gly Gly Asp Gly Val Ser Asp Ala Lys Lys His Ser Gln Ser
                50                  55                  60
    Val Arg Gln Gly Leu Ala Gly Ile Ile Asp Leu Pro Ser Glu Ala Pro
10  65                  70                  75                  80
    Ser Glu Val Asp Ile Ser His Gly Ser Glu Asp Pro Arg Gly Pro Thr
                        85                  90                  95
    Asp Ser Tyr Gln Met Asn Gly Ile Ile Asn Glu Thr His Asn Gly Arg
                    100                 105                 110
15  His Ala Ser Val Ser Lys Val Val Glu Phe Cys Ala Ala Leu Gly Gly
                    115                 120                 125
    Lys Thr Pro Ile His Ser Ile Leu Val Ala Asn Asn Gly Met Ala Ala
                    130                 135                 140
    Ala Lys Phe Met Arg Ser Val Arg Thr Trp Ala Asn Asp Thr Phe Gly
20  145                 150                 155                 160
    Ser Glu Lys Ala Ile Gln Leu Ile Ala Met Ala Thr Pro Glu Asp Met
                    165                 170                 175
    Arg Ile Asn Ala Glu His Ile Arg Ile Ala Asp Gln Phe Val Glu Val
                    180                 185                 190
25  Pro Gly Gly Thr Asn Asn Asn Asn Tyr Ala Asn Val Gln Leu Ile Val
                    195                 200                 205
    Gly Met Ala Gln Lys Leu Gly Val Ser Ala Val Trp Pro Gly Trp Gly
                210                 215                 220
    His Ala Ser Glu Asn Pro Glu Leu Pro Asp Ala Leu Thr Ala Lys Gly
30  225                 230                 235                 240
    Ile Val Phe Leu Gly Pro Pro Ala Ser Ser Met Asn Ala Leu Gly Asp
                    245                 250                 255
    Lys Val Gly Ser Ala Leu Ile Ala Gln Ala Ala Gly Val Pro Thr Leu
                260                 265                 270
35  Ala Trp Ser Gly Ser His Val Glu Val Pro Leu Glu Cys Cys Leu Asp
                275                 280                 285
    Ala Ile Pro Glu Glu Met Tyr Arg Lys Ala Cys Val Thr Thr Thr Glu
                290                 295                 300
    Glu Ala Val Ala Ser Cys Gln Val Val Gly Tyr Pro Ala Met Ile Lys
40  305                 310                 315                 320
```

8

Ala Ser Trp Gly Gly Gly Gly Lys Gly Ile Arg Lys Val His Asn Asp
              325   330   335

Asp Glu Val Arg Ala Leu Phe Lys Gln Val Gln Gly Glu Val Pro Gly
              340   345   350

5 Ser Pro Ile Phe Val Met Arg Leu Ala Ser Gln Ser Arg His Leu Glu
              355   360   365

Val Gln Leu Leu Cys Asp Gln Tyr Gly Asn Val Ala Ala Leu His Ser
         370   375   380

Arg Asp Cys Ser Val Gln Arg Arg His Gln Lys Ile Ile Glu Glu Gly
10 385   390   395   400

Pro Val Thr Val Ala Pro Arg Glu Thr Val Lys Ala Leu Glu Gln Ala
                   405   410   415

Ala Arg Arg Leu Ala Lys Ala Val Gly Tyr Val Gly Ala Ala Thr Val
              420   425   430

15 Glu Tyr Leu Tyr Ser Met Glu Thr Gly Asp Tyr Tyr Phe Leu Glu Leu
              435   440   445

Asn Pro Arg Leu Gln Val Glu His Pro Val Thr Glu Trp Ile Ala Glu
         450   455   460

Val Asn Leu Pro Ala Ala Gln Val Ala Val Gly Met Gly Ile Pro Leu
20 465   470   475   480

Trp Gln Ile Pro Glu Ile Arg Arg Phe Tyr Gly Met Asp Tyr Gly Gly
              485   490   495

Gly Tyr Asp Ile Trp Arg Lys Thr Ala Ala Leu Ala Thr Pro Phe Asn
              500   505   510

25 Phe Asp Glu Val Asp Ser Gln Trp Pro Lys Gly His Cys Val Ala Val
              515   520   525

Arg Ile Thr Ser Glu Asp Pro Asp Asp Gly Phe Lys Pro Thr Gly Gly
         530   535   540

Lys Val Lys Glu Ile Ser Phe Lys Ser Lys Pro Asn Val Trp Ala Tyr
30 545   550   555   560

Phe Ser Val Lys Ser Gly Gly Gly Ile His Glu Phe Ala Asp Ser Gln
              565   570   575

Phe Gly His Ala Phe Ala Tyr Gly Leu Ser Arg Pro Ala Ala Ile Thr
              580   585   590

35 Asn Met Ser Leu Ala Leu Lys Glu Ile Gln Ile Arg Gly Glu Ile His
              595   600   605

Ser Asn Val Asp Tyr Thr Val Asp Leu Leu Asn Ala Ser Asp Phe Arg
         610   615   620

Glu Asn Lys Ile His Thr Gly Trp Leu Asp Thr Arg Ile Ala Met Arg
40 625   630   635   640

9

Val Gln Ala Glu Arg Pro Pro Trp Tyr Ile Ser Val Val Gly Gly Ala
              645   650   655

Leu Tyr Lys Thr Val Thr Thr Asn Ala Ala Thr Val Ser Glu Tyr Val
              660   665   670

5 Ser Tyr Leu Thr Lys Gly His Ile Pro Pro Lys His Ile Ser Leu Val
              675   680   685

Asn Ser Thr Val Asn Leu Asn Ile Glu Gly Ser Lys Tyr Thr Ile Glu
             690   695   700

Thr Val Arg Thr Gly His Gly Ser Tyr Arg Leu Arg Met Asn Asp Ser
10 705   710   715   720

Thr Val Glu Ala Asn Val Gln Ser Leu Cys Asp Gly Gly Leu Leu Met
              725   730   735

Gln Leu Asp Gly Asn Ser His Val Ile Tyr Ala Glu Glu Glu Ala Gly
              740   745   750

15 Gly Thr Arg Leu Gln Ile Asp Gly Lys Thr Cys Leu Leu Gln Asn Asp
              755   760   765

His Asp Pro Ser Lys Leu Leu Ala Glu Thr Pro Cys Lys Leu Leu Arg
             770   775   780

Phe Leu Val Ala Asp Gly Ala His Val Asp Ala Asp Val Pro Tyr Ala
20 785   790   795   800

Glu Val Glu Val Met Lys Met Cys Met Pro Leu Leu Ser Pro Ala Ser
              805   810   815

Gly Val Ile His Cys Met Met Ser Glu Gly Gln Ala Leu Gln Ala Gly
              820   825   830

25 Asp Leu Ile Ala Arg Leu Asp Leu Asp Asp Pro Ser Ala Val Lys Arg
              835   840   845

Ala Glu Pro Phe Asp Gly Ile Phe Pro Gln Met Glu Leu Pro Val Ala
             850   855   860

Val Ser Ser Gln Val His Lys Arg Tyr Ala Ala Ser Leu Asn Ala Ala
30 865   870   875   880

Arg Met Val Leu Ala Gly Tyr Glu His Asn Ile Asn Glu Val Val Gln
              885   890   895

Asp Leu Val Cys Cys Leu Asp Asn Pro Glu Leu Pro Phe Leu Gln Trp
              900   905   910

35 Asp Glu Leu Met Ser Val Leu Ala Thr Arg Leu Pro Arg Asn Leu Lys
              915   920   925

Ser Glu Leu Glu Asp Lys Tyr Lys Glu Tyr Lys Leu Asn Phe Tyr His
              930   935   940

Gly Lys Asn Glu Asp Phe Pro Ser Lys Leu Leu Arg Asp Ile Ile Glu
40 945   950   955   960

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   Glu Asn Leu Ser Tyr Gly Ser Glu Lys Glu Lys Ala Thr Asn Glu Arg
                   965                 970                 975
   Leu Val Glu Pro Leu Met Asn Leu Leu Lys Ser Tyr Glu Gly Gly Arg
                   980                 985                 990
 5 Glu Ser His Ala His Phe Val Val Lys Ser Leu Phe Glu Glu Tyr Leu
                   995                 1000                1005
   Thr Val Glu Glu Leu Phe Ser Asp Gly Ile Gln Ser Asp Val Ile Glu
               1010                1015                1020
   Thr Leu Arg His Gln His Ser Lys Asp Leu Gln Lys Val Val Asp Ile
10 1025                1030                1035                1040
   Val Leu Ser His Gln Gly Val Arg Asn Lys Ala Lys Leu Val Thr Ala
                   1045                1050                1055
   Leu Met Glu Lys Leu Val Tyr Pro Asn Pro Gly Gly Tyr Arg Asp Leu
                   1060                1065                1070
15 Leu Val Arg Phe Ser Ser Leu Asn His Lys Arg Tyr Tyr Lys Leu Ala
                   1075                1080                1085
   Leu Lys Ala Ser Glu Leu Leu Glu Gln Thr Lys Leu Ser Glu Leu Arg
                   1090                1095                1100
   Ala Ser Val Ala Arg Ser Leu Ser Asp Leu Gly Met His Lys Gly Glu
20 1105                1110                1115                1120
   Met Ser Ile Lys Asp Asn Met Glu Asp Leu Val Ser Ala Pro Leu Pro
                   1125                1130                1135
   Val Glu Asp Ala Leu Ile Ser Leu Phe Asp Tyr Ser Asp Arg Thr Val
                   1140                1145                1150
25 Gln Gln Lys Val Ile Glu Thr Tyr Ile Ser Arg Leu Tyr Gln Pro His
                   1155                1160                1165
   Leu Val Lys Asp Ser Ile Gln Met Lys Phe Lys Glu Ser Gly Ala Ile
                   1170                1175                1180
   Thr Phe Trp Glu Phe Tyr Glu Gly His Val Asp Thr Arg Asn Gly His
30 1185                1190                1195                1200
   Gly Ala Ile Ile Gly Gly Lys Arg Trp Gly Ala Met Val Val Leu Lys
                   1205                1210                1215
   Ser Leu Glu Ser Ala Ser Thr Ala Ile Val Ala Ala Leu Lys Asp Ser
                   1220                1225                1230
35 Ala Gln Phe Asn Ser Ser Glu Gly Asn Met Met His Ile Ala Leu Leu
                   1235                1240                1245
   Ser Ala Glu Asn Glu Ser Asn Ile Ser Gly Ile Ser Ser Asp Asp Gln
                   1250                1255                1260
   Ala Gln His Lys Met Glu Lys Leu Ser Lys Ile Leu Lys Asp Thr Ser
40 1265                1270                1275                1280
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11

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       Val Ala Ser Asp Leu Gln Ala Ala Gly Leu Lys Val Ile Ser Cys Ile
                       1285                1290                1295
       Val Gln Arg Asp Glu Ala Arg Met Pro Met Arg His Thr Phe Leu Trp
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   5   Leu Asp Asp Lys Ser Cys Tyr Glu Glu Glu Gln Ile Leu Arg His Val
                       1315                1320                1325
       Glu Pro Pro Leu Ser Thr Leu Leu Glu Leu Asp Lys Leu Lys Val Lys
                       1330                1335                1340
       Gly Tyr Asn Glu Met Lys Tyr Thr Pro Ser Arg Asp Arg Gln Trp His
  10   1345                1350                1355                1360
       Ile Tyr Thr Leu Arg Asn Thr Glu Asn Pro Lys Met Leu His Arg Val
                       1365                1370                1375
       Phe Phe Arg Thr Ile Val Arg Gln Pro Asn Ala Gly Asn Lys Phe Arg
                       1380                1385                1390
  15   Ser Ala Gln Ile Ser Asp Ala Glu Val Gly Cys Pro Glu Glu Ser Leu
                       1395                1400                1405
       Ser Phe Thr Ser Asn Ser Ile Leu Arg Ser Leu Met Thr Ala Ile Glu
                       1410                1415                1420
       Glu Leu Glu Leu His Ala Ile Arg Thr Gly His Ser His Met Tyr Leu
  20   1425                1430                1435                1440
       Cys Ile Leu Lys Glu Gln Lys Leu Leu Asp Leu Ile Pro Phe Ser Gly
                       1445                1450                1455
       Ser Thr Ile Val Asp Val Gly Gln Asp Glu Ala Thr Ala Cys Ser Leu
                       1460                1465                1470
  25   Leu Lys Ser Met Ala Leu Lys Ile His Glu Leu Val Gly Ala Arg Met
                       1475                1480                1485
       His His Leu Ser Val Cys Gln Trp Glu Val Lys Leu Lys Leu Asp Cys
                       1490                1495                1500
       Asp Gly Pro Ala Ser Gly Thr Trp Arg Val Val Thr Thr Asn Val Thr
  30   1505                1510                1515                1520
       Gly His Thr Cys Thr Ile Asp Ile Tyr Arg Glu Val Glu Glu Ile Glu
                       1525                1530                1535
       Ser Gln Lys Leu Val Tyr His Ser Ala Ser Ser Ser Ala Gly Pro Leu
                       1540                1545                1550
  35   His Gly Val Ala Leu Asn Asn Pro Tyr Gln Pro Leu Ser Val Ile Asp
                       1555                1560                1565
       Leu Lys Arg Cys Ser Ala Arg Asn Asn Arg Thr Thr Tyr Cys Tyr Asp
                       1570                1575                1580
       Phe Pro Leu Ala Phe Glu Thr Ala Leu Gln Lys Ser Trp Gln Ser Asn
  40   1585                1590                1595                1600
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12

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     Gly Ser Thr Val Ser Glu Gly Asn Glu Asn Ser Lys Ser Tyr Val Lys
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     Ala Thr Glu Leu Val Phe Ala Glu Lys His Gly Ser Trp Gly Thr Pro
                   1620                1625                1630
  5  Ile Ile Pro Met Glu Arg Pro Ala Gly Leu Asn Asp Ile Gly Met Val
                   1635                1640                1645
     Ala Trp Ile Met Glu Met Ser Thr Pro Glu Phe Pro Asn Gly Arg Gln
                   1650                1655                1660
     Ile Ile Val Val Ala Asn Asp Ile Thr Phe Arg Ala Gly Ser Phe Gly
 10  1665                1670                1675                1680
     Pro Arg Glu Asp Ala Phe Phe Glu Thr Val Thr Asn Leu Ala Cys Glu
                   1685                1690                1695
     Arg Lys Leu Pro Leu Ile Tyr Leu Ala Ala Asn Ser Gly Ala Arg Ile
                   1700                1705                1710
 15  Gly Ile Ala Asp Glu Val Lys Ser Cys Phe Arg Val Gly Trp Ser Asp
                   1715                1720                1725
     Glu Gly Ser Pro Glu Arg Gly Phe Gln Tyr Ile Tyr Leu Thr Glu Glu
                   1730                1735                1740
     Asp Tyr Ala Arg Ile Ser Ser Ser Val Ile Ala His Lys Leu Glu Leu
 20  1745                1750                1755                1760
     Asp Ser Gly Glu Ile Arg Trp Ile Ile Asp Ser Val Val Gly Lys Glu
                   1765                1770                1775
     Asp Gly Leu Gly Val Glu Asn Ile His Gly Ser Ala Ala Ile Ala Ser
                   1780                1785                1790
 25  Ala Tyr Ser Arg Ala Tyr Glu Glu Thr Phe Thr Leu Thr Phe Val Thr
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     Gly Arg Thr Val Gly Ile Gly Ala Tyr Leu Ala Arg Leu Gly Ile Arg
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     Cys Ile Gln Arg Leu Asp Gln Pro Ile Ile Leu Thr Gly Phe Ser Ala
 30  1825                1830                1835                1840
     Leu Asn Lys Leu Leu Gly Arg Glu Val Tyr Ser Ser His Met Gln Leu
                   1845                1850                1855
     Gly Gly Pro Lys Ile Met Ala Thr Asn Gly Val Val His Leu Thr Val
                   1860                1865                1870
 35  Pro Asp Val Leu Glu Gly Val Ser Asn Ile Leu Arg Trp Leu Ser Tyr
                   1875                1880                1885
     Val Pro Ala Asn Ile Gly Gly Pro Leu Pro Ile Thr Lys Pro Leu Asp
                   1890                1895                1900
     Pro Pro Asp Arg Pro Val Ala Tyr Ile Pro Glu Asn Thr Cys Asp Pro
 40  1905                1910                1915                1920
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13

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   Arg Ala Ala Ile Cys Gly Val Asp Asp Ser Gln Gly Lys Trp Leu Gly
                 1925              1930               1935
   Gly Met Phe Asp Lys Asp Ser Phe Val Glu Thr Phe Glu Gly Trp Ala
                 1940              1945               1950
 5 Lys Thr Val Val Thr Gly Arg Ala Lys Leu Gly Gly Ile Pro Val Gly
                 1955              1960               1965
   Val Ile Ala Val Glu Thr Gln Thr Met Met Gln Ile Ile Pro Ala Asp
               1970              1975              1980
   Pro Gly Gln Leu Asp Ser His Glu Arg Ser Val Pro Arg Ala Gly Gln
10 1985              1990              1995               2000
   Val Trp Phe Pro Asp Ser Ala Thr Lys Thr Ala Gln Ala Leu Leu Asp
                 2005              2010               2015
   Phe Asn Arg Glu Gly Leu Pro Leu Phe Ile Leu Ala Asn Trp Arg Gly
                 2020              2025               2030
15 Phe Ser Gly Gly Gln Arg Asp Leu Phe Glu Gly Ile Leu Gln Ala Gly
                 2035              2040               2045
   Ser Thr Ile Val Glu Asn Leu Arg Thr Tyr Asn Gln Pro Ala Phe Val
                 2050              2055               2060
   Tyr Ile Pro Met Ala Gly Glu Leu Arg Gly Gly Ala Trp Val Val Val
20 2065              2070              2075               2080
   Asp Ser Lys Ile Asn Pro Asp Arg Ile Glu Cys Tyr Ala Glu Arg Thr
                 2085              2090               2095
   Ala Lys Gly Asn Val Leu Glu Pro Gln Gly Leu Ile Glu Ile Lys Phe
                 2100              2105               2110
25 Arg Ser Glu Glu Leu Gln Asp Cys Met Gly Arg Leu Asp Pro Glu Leu
                 2115              2120               2125
   Ile Asn Leu Lys Ala Lys Leu Gln Asp Val Asn His Gly Asn Gly Ser
                 2130              2135               2140
   Leu Pro Asp Ile Glu Gly Ile Arg Lys Ser Ile Glu Ala Arg Thr Lys
30 2145              2150              2155               2160
   Gln Leu Leu Pro Leu Tyr Thr Gln Ile Ala Ile Arg Phe Ala Glu Leu
                 2165              2170               2175
   His Asp Thr Ser Leu Arg Met Ala Ala Lys Gly Val Ile Lys Lys Val
                 2180              2185               2190
35 Val Asp Trp Glu Glu Ser Arg Ser Phe Phe Tyr Lys Arg Leu Arg Arg
                 2195              2200               2205
   Arg Ile Ala Glu Asp Val Leu Ala Lys Glu Ile Arg Gln Ile Val Gly
               2210              2215              2220
   Asp Lys Phe Thr His Gln Leu Ala Met Glu Leu Ile Lys Glu Trp Tyr
40 2225              2230              2235               2240
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14

    Leu Ala Ser Gln Ala Thr Thr Gly Ser Thr Gly Trp Asp Asp Asp Asp
                     2245   2250   2255
    Ala Phe Val Ala Trp Lys Asp Ser Pro Glu Asn Tyr Lys Gly His Ile
                     2260   2265   2270
  5 Gln Lys Leu Arg Ala Gln Lys Val Ser His Ser Leu Ser Asp Leu Ala
                     2275   2280   2285
    Asp Ser Ser Ser Asp Leu Gln Ala Phe Ser Gln Gly Leu Ser Thr Leu
                     2290   2295   2300
    Leu Asp Lys Met Asp Pro Ser Gln Arg Ala Lys Phe Val Gln Glu Val
 10 2305   2310   2315   2320
    Lys Lys Val Leu Asp
                     2325

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<221> unsure

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ctcsgcttac gcaattgttt gtggcagcwg cgtgggctaa atgtargttg tctcttgttg    180
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tgtggtttat tcagggatgc ccatgcccat gctagattga taggtgccat tctaatggta    300
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agcacacctt ttcctttgtg tgggtgcata aggaatgcac ttggcttcgt tccctgatag    420
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ggtggttgtg catctttytg ctycccctgg ytgtctaata cctgcatgta actgatgacc    540
yycytttatg tatcatatag attacatcct tttgttgtac atctcaattc tgaaaaaaca    600
atgttttgca ttcttagcgc tctgtgcaca aggaaaagga ggttttacct gcaacttttt    660
ttttcgagaa aaaacaaacc tttctgaaag gcagtgatca tttagtataa agaaaatttg    720
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     atttactttc ttcagagaga atatkccaar caaacaattt tcttactgtc tgagccacga        780

     aatttgatct tgatcttact ttcacaagcc acatgaagcc ttatcatcgc tctgataaaa        840

     aarccaaata ggtgattcat agaatgagar aaagaacctg ttgccatttg gggaccttgt        900

     tgtgtactca ttatcccccc tgctcaggtt gaggtttcct tgccactgcc accccttggc        960

  5  cccttcttat acaaccatct ccattgaaaa agattttgca ctacatttgg gcttcgtatg       1020

     acaaaaaagg aaaataaaac taaacagcag aaacatagta taattatagg taaaaggttc       1080

     tggcaagttt gagtggaaga gacctttgta tatttggaca tatttcacta gtaaatagtt       1140

     ttctaaaatc ttcatgaatg gtggccaata aacttgataa gatctcaaca tggcaggttc       1200

     cttcmaaatg agaggaaaac tggaaacatc acaaatattt tttagcgagt ggcctataaa       1260

  10 ttataatgtt gctttcattt ctttgatatt caaaacttcc taagagtatt ctgctagagc       1320

     tctgatggtg tcttttgcct ctgtcagatt ttccaggagt tttcttccct ttttatggca       1380

     ctgtgcgttt gagaaggtct tcaattgtgc tgtctgggcc acggaacgac aatgtcacag       1440

     cttggattag ccgcagctgc ctcaaaggcc ttgccactac tccctaatcg ccagagaagt       1500

     tcagctggga ctacattctc atcatcttca ttatcgaggc ccttaaacag aaggaaaagc       1560

  15 catactcgtt cactccgtga tggcggagat ggggtatcag atgccaaaaa gcacagccag       1620

     tctgttcgtc aaggtactgt gaatatcttt tgatacaagc taaaattttg ctacagaata       1680

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     tctagttgga ttggttaggt ggsctgaata ccactcctta aggtcttgag tttgcttttc       1800

     cccncggagc gaattttagg ctagggttac ccccccaccc ccacccgaat ctgcacagyc       1860

  20 cggycgyggy cgycctcata taggctacga tgtcattgtg tatcggcggg ccaggggttt       1920

     aagagttttc ttgacctttg ttagaagatc ttaataatac aatgtccaag ggctgtctta       1980

     ccctgtaggt cgagttttta gttgttttaa catggtaatg tttgaagcct cattctaggt       2040

     rccaatatag atatgctcac tgctcagttt caaatgtttg tctgcatgta ggtcttgctg       2100

     gcattatcga cctcccaagt gaggcacctt ccgaagtgga tatttcacag taaggactac       2160

  25 aatattttgc gtacgtttgt tttggaaaaa gaaaatattc tcagcttatt tatactagct       2220

     tcgctaatac tgaaatgctg tcttaatgtc ctggtgctgt atgctcaatc tttcatagta       2280

     aatgctgcaa aatatgtgat gtaactgttg caacacagcc agggacctgt tatttagagc       2340

     atggtgaatg ctctggttca gttatatgat gtagttatag ctcatgttga agaattagtt       2400

     gcagtgtttg ctggacaatg gtcacttatt ataaatcata tctgcataca catttgtgac       2460

  30 ttctgttgct gtaaatgccc gcatttttg agaaaaattt aaatgcttgg cctaaattgg       2520

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     aagacatgcc tcagtgtcca aggttgttga attttgtgcg gcactaggtg gcaaaacacc       2760

  35 aattcacagt atattagtgg ccaacaatgg aatggcagca cccaaattta tgaggagtgt       2820

     ccggacatgg gctaatgata cttttggatc tgagaaggca attcaactca tagctatggc       2880

     aactccggaa gacatgagga taaatgcaga acacattaga attgctgacc aattacgtag       2940

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     gccttgctaa tctgttagtt tactactggt ctgctgtttc ctttatttgt tgtataatga       3060

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17

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     catcattaac tgtgaaatat tgcagatggc acaaaaacta ggtgtttctg ctgtttggcc      3180
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     cgttttttctt ggcccacctg satcatcaat gaatgctttg ggagataagg tcggctcagc     3300
     tctcattgct caagcagccg gggncccaac tcttgcttgg agtggatcac atgtgagtct      3360
   5 cactctttga ttactatccg cctgtctcat tgctctctct ttcatattct aatgacacta      3420
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     tgctagtctt tgctcatgtg tcattctacc aagtgggtta ctgtaacatt gcactctatg       420
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     acgaaatttg atcttgatct tactttcaca agccacatga agccttatca tcgctctgat       780
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     tataacaaaa aaggaaaata aaactaaaca gcagaaacat agtataatta taggtaaaag      1020
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     agttttctaa aatgttcatg aatggtggcc aataaacttg ataagatctc aacatggcag      1140
     gttccttcaa aatgagagga aaactggaaa catcacaaat attttttagc gagtggccta      1200
  40 taaattataa tgttgctttc atttctttga tattcaaaac ttcctaagag tattctgcta      1260
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18

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40  tattcactgt gatctttgtg tcgtgtgtga gttgttgatc cttcccttgc tccgtgattc     180
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21

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     tttgtgaaat cttttgaaag ggcgagaggc tccaagctgt ggagattcct cgcaagtggg    240
     attaagaaaa gcaaagcaac accgtggtat tcaagttggt ctttggaccg cttgagaggg    300
     gttgattgca accctcgtcc gttgggacgc cacaacgtgg agtaggcaag cgttggtctt    360
     ggccgaacca cgggataacc accgtgccat ctctgtgatt gatatctctt ggttattgtg    420
  5  ttgtgttgag atccttctct agccacttgg caaattactg tgctaacaat taatcaagtt    480
     ttgtggctta agattttgaa gtattacagg atctgcatca tggtctgtgt ctccacagct    540
     atgacaccca caggaattca tgtgttcctt ggagccactc ttggatgacc taaaggaatt    600
     atttctaacc ggcttgtaca catatgatgc atcaagagat gagtgtttta ctatgcgagg    660
     ggccatgctt atgaccataa gtaatcttcc tggtttagaa atgcttgctt ctcatatggt    720
 10  tcatgggaaa ttcgcatgcc tccttgtggt gaaaatgtct ggacaaaaca gctgaagaat    780
     ggtcgtaaat cttgttttat gggaaatcgc caatatattg atcttgatca ttcttattgc    840
     ttggatgcag actccgtttg atggaacgat agacttcgaa caaaacctaa aacctattat    900
     gatcgtccaa ttttggatga aatcatcaca cttggtgatt tcaagaactc aaaaayttac    960
     agttaattgg atataggagg gngcaaaaac acagtaagtt ggacattcca taaggggatt   1020
 15  tattttagtt gacaataaag tagatgggca tcatcctgag tttngtttgg catcgtgtcg   1080
     tagattgaaa ctgtaaggat ggacatggta gntaacaggt tgagatgaat gattcaacag   1140
     ttgaagcgaa tgtacaatct ttatgtgatg gtggctntta atgcaggtaa ctagtttttt   1200
     tttatgcttt attattaatt agttggataa atggtttnga tttntgattg ttaaantgca   1260
     atggctccag ttggatggna acagccangt aatttatgca gaagnagnag ntggtggtac   1320
 20  acggnttcag attgatggaa aganatgttt attgcaggta aatantccct tnttccttta   1380
     tattttgtt gtntgattgt ataantttgn tagattattt gtataattta ttattgcatt    1440
     tcaccccact aanttatttt taaaagatgg gttttgttgt ttgnttcagc nggcgacatc   1500
     acataagnaa attgtgatta atttttgttt ttttgcagna tgaccatgat ccatcaaagt   1560
     tatta                                                               1565
 25
          <210> 15
          <211> 1168
          <212> DNA
          <213> Zea mays
 30
          <220>
          <221> unsure
          <222> (60)...(60)
          <223> "n" represents "a" or "g" or "c" or "t"
 35
          <221> unsure
          <222> (1024)...(1024)
          <223> "n" represents "a" or "g" or "c" or "t"
 40
          <221> unsure
```

22

<400> 15

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acataagctg ggttagtagt ggtgaattag tggattattg atctgttgtg ggcaaggagn      60
tggcttggtg tcgagaaaaa acatggaagt gtgtattgcc agtgcttatt ytrgggatat     120
gagggaattw amattacatt tgtgactggg cggactgtag gataggagtt atcttgytcg     180
attggtatac ggtgcataca gskyttgacc agctattatt ttaacagggt tttctgccct     240
gaacaagtcc ttgggcggga agtgtacagc tcccacatgc agcttggtgg tcctaagatc     300
atggcgacca atggtgttgt ccacctcact gttccagatg accttgaagg tgtttccaat     360
atattgaggt ggctcagcta tgttcctgca aacattggtg gacctcttcc tattaccaaa     420
cctctggacc ctccagacag acctgttgct tacatccctg agaacacatg cgatccacgt     480
gcagctatct gtggtgtaga tgacagccaa gggaaatggt tgggtggtat gtttgacaaa     540
gacagctttg tggagacatt tgaaggatgg gcaaaaacag tggttactgg cagagcaaag     600
cttggaggaa ttccatgcat cttaataaac acagttggcc cttaaagcaa gtgaacttct     660
tgaacaaacc aaactaagtg aactccgtgc aagcgttgca agaagccttt cggatctggg     720
gatgcataag ggagaaatga gtattaagga taacatggaa gatttagtct ctgccccatt     780
acctgttgaa gatgctctga tttctttgtt tgattacagt gatcgaactg ttcagcagaa     840
agtgattgag acatacatat cacgattgta ccaggtatta tatcaactaa cttaatgtct     900
tccatagtct cactaagcat atctgatatg tttagatacc ctacatggaa tgctcatctt     960
ttcatttgac acaaagaaac attgagaaat gagatgctga cgattggctg aaattaactg    1020
ggtntgagaa attgtgatct cccaacttgt taatgcacaa tgttctggct aacttgccaa    1080
tattttttca gcctcatctt gttnaggata gcanccaaat gaaatccaag gatctggtgc    1140
tattactttt tgggaattta tgaagggc                                      1168
```


<210> 16

<211> 638

<212> DNA

<213> Zea mays

<220>

<221> unsure

<222> (358)...(358)

<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure

23

```
<222> (398)...(398)
<223> "n" represents "a" or "g" or "c" or "t"


<400> 16

ctcccaatat tgtcatgagg cttgcatccc aggttagttt tttttccttt ctgaaattta    60
tattccatac cttttcacct ttagttatcc ttgtattttc tggaagcttc atctgatgca   120
ttattgacaa atgcactaat ggtcatcata tttggagatt aacatattta tcttaattga   180
tgggaactct tgaaaatgac aatggttgag cagataatta acagtttttt aataaaaaaa   240
catgcatttc taggagttgg actaagcttt tcttagtatg aagtgccatg ttttacatgg   300
tccatttgtg tcaatttaca gtcggtatca tggaaaggtt gtcataatgg ctggaganaa   360
acaacacatc ttgtttctca acacttgtgg gagaagangt tttacctttt ttcctaaaat   420
tactttttgt actaaattgt ataatttttc caatattctc catgattatt gaactctgct   480
gtgttcaaac agccaaaaca tgtttccata ctttacacct ttatttttta gatggaacct   540
ggaattgtgc tctgttatct gtatcatgca tatattgatc ttaaacctat ctctattgta   600
gaatccgcac ttgaattcag ttgcttgtga tcaatatg                          ·638



<210> 17
<211> 558
<212> DNA
<213> Zea mays


<220>
<221> unsure
<222> (41)...(41)
<223> "n" represents "a" or "g" or "c" or "t"


<221> unsure
<222> (103)...(103)
<223> "n" represents "a" or "g" or "c" or "t"


<400> 17

ggtaaccacc acaccgcgg cgcttaatgg ccgtacaggg nggtcccatt cgccattcag    60
gtgcgcaact gttgggaagg gcgatcggtg cgggcttctt cgntattacg ccagctggcg   120
aaaggggat gtgctgcaag gcgattaagt tgggtaacgc cagggttttc ccagtcacga   180
cgttgtaaaa cgacggccag tgagcgcgcg taatacgact cactataggg cgaattgggt   240
accgggcccc ccctcgaggt cgacctgcag gtcaacggat cctaggggc caacagattc   300
ttatcaaatg aatgggatta tcaatgaaac acataatgga agacatgcct cagtgtccaa   360
ggttgttgaa ttttgtgcgg cactaggtgg caaaacacca attcacagta tattagtggc   420
caacaatgga atggcagcag caaaatttat gaggagtgtc cggacatggg ctaatgatac   480
ttttggatct gagaaggcaa ttcaactcat agctatggca actccggaag acatgaggta   540
```

24

aatgcagaac acattaga   558

                                                        <210> 18
                                                        <211> 975

5   <212> DNA
                                                        <213> Zea mays

                                                        <220>
                                                        <221> unsure

10   <222> (784)...(784)
                                                        <223> "n" represents "a" or "g" or "c" or "t"

                                                        <400> 18
            gaataatctg cctgcagctc aagttgctgt tggaatgggc atacctcttt ggcagattcc   60

15 aggtaattac caatttacca acttatttag ttccttattg ttttattctc taattttcta   120

   cttatgtaga aatcagacgt ttctatggaa tggactatgg aggagggtat gacatttgga   180

   ggaaaacagc agctcttgct acaccattta attttgatga agtagattct caatggccaa   240

   agggccattg tgtagcagtt agaattacta gtgaggaccc agatgatggt ttcaaaccta   300

   ctggtgggaa agtgaaggta agttttctag atgacatgta ttatatatcg ttcaaagaga   360

20 ttaagtttgg ttaaatgact aggtcttgat tttttatctt tcaggagata agttttaaaa   420

   gcaagcctaa tgtttgggcc tacttctcag taaaggtaac ttgttaactt tagtacgctg   480

   tcacattatt cttcttgtg aaaataattt gaacggttct ctttgtattt taaccatcca   540

   tcgtctcatt tascagagca cacaaatatt tgcactgacc ccctcccct tatctgcttt   600

   cagtctggtg gaggcattca tgaatttgct gattctcagt tcggtatgtg taaaccaaga   660

25 gtattctttg taatttatat tggtcctcaa ttttgaaata ttgctctttc cgttacagga   720

   cawgtttttg catatgggct ctctagatca gcagcaataa caaacatgac tcttgcatta   780

   aaanagattc aaattcgtgg agaaattcat tcaaatgttt gattacacag ttgatctctt   840

   aaatgttaag aaatattaac cacctttaa atcacatttt ccattatgtt tgattccata   900

   tcattaattt tgattttcta ttatggctaa acctgtggtg ctattttcct attatcccag   960

30 gcttccgact ttaga   975

                                                          <210> 19
                                                        <211> 852
                                                        <212> DNA

35   <213> Zea mays

                                                        <400> 19
            ggatcctagg gggccaacag attcttatca aatgraatgg gattatcaat gaaacacata   60

   atggaagaca tgcctcagtg tccaaggttg ttgaattttg tgcggcacta ggtggcaaaa   120

40 caccaattca cagtatatta gtggccaaca atggaatggc agcagcaaaa tttatgagga   180

25

```
    gtgtccggac atgggctaat gatacttttg gatctgagaa ggcaattcaa ctcatagcta    240
    tggcaactcc ggaagacatg aggataaatg cagaacacat tagaattgct gaccaattac    300
    gtagargtgc ctggtggaac aaacaataat aactacgcca atgttcaact catagtggaa    360
    gttagccttg ctaatctgtt agtttactac tggtctgctg tttcctttat ttgttgtata    420
5   atgattgaca tatttaagta gagaaattta tatttctcct ctgctgttgt ggaagtccaa    480
    ttgtcaccat taactgtgaa atattgcaga tggcacaaaa actaggtgtt tctgctgttt    540
    ggcctggttg gggtcatgct tctgagaatc ctgaactgcc agatgcattg accgcaaaag    600
    ggatcgtttt tcttggccca cctgcatcat caatgaatgc tttgggagat aaggtcggct    660
    cagctctcat tgctcaagca gccggggtcc caactcttgc ttggagtgga tcacatgtga    720
10  gtctcactct ttgattacta tccgcctgtc tcattgctct ctctttcata ttctaatgac    780
    actaaattta ggttgaagtt ccattagagt gctgcttaga cgcgatacct gaggagatgt    840
    atagaaaagc tt                                                        852
```

```
    <210> 20
15  <211> 231
    <212> DNA
    <213> Zea mays


    <220>
20  <221> unsure
    <222> (125)...(125)
    <223> "n" represents "a" or "g" or "c" or "t"


    <400> 20
25  aattcctgtg ggtgttatag ctgtggagac acagaccatg atgcagctca tccctgctga     60
    tccaggtcaa cttgattccc atgagcgatg tgttcctcgg ctggacaag tgtggttccc      120
    agatnctgca accaagacag ctcaggcatt attagacttc aaccgtgaag gattgcctct     180
    gttcatcctg ctaactgga gaggcttctc tgggggacag agagatctct t              231
```

```
30  <210> 21
    <211> 207
    <212> DNA
    <213> Zea mays


35  <220>
    <221> unsure
    <222> (165)...(165)
    <223> "n" represents "a" or "g" or "c" or "t"


40  <400> 21
```

26

```
aattcatgca tcttaataaa cacagttggc ccttaaagca agtgaacttc ttgaacaaac      60
caaactaagt gaactctgtt ccagcattgc aagaagcctt tcagatctgg ggatgcataa     120
gggagaaatg actattaagg atagcatgga agatttagtc tctgncccat tgcctgttga     180
agatgctctt atttctttgt ttgatta                                         207
```

5

```
        <210> 22
        <211> 180
        <212> DNA
        <213> Zea mays
```

10

```
        <220>
        <221> unsure
        <222> (59)...(59)
        <223> "n" represents "a" or "g" or "c" or "t"
```

15

```
        <400> 22
    atagacctgt cgcatacatc cctgagaaca catgcgatcc gcgtgcagcc atccgtggng      60
    tagatgacag ccaagggaaa tggttgggtg gtatgtttga caaagacagc tttgtggaga     120
    catttgaagg atgggcaaaa acagtggtta ctggtagagc aaagcttgga ggaaggaatt     180
```

20

```
        <210> 23
        <211> 3031
        <212> DNA
        <213> Avena sativa
```

25

```
        <400> 23
    acaaaatagt aaagacctcc agaaggttgt agacattgtt ttgtctcacc agggtgtgag      60
    aagcaaaacc aagctgatac tcacgctcat ggagaaactg gtctatccaa accctgctgc     120
    ctatagggac cagctgattc gcttttcttc cctcaaccat aaaagatatt acaagctggc     180
 30 ccttaaagct agtgaacttc tcgaacaaac caagctcagt gaactccgca caagcattgt     240
    aaggaacctt tcagcgctgg agatgtttac tgaagaaagg gcaggtttct cgttgcaaga     300
    gaagaaattg gccattaatg agagcatggg agatttagtc agtgccccac tgccagttga     360
    agatgcactt atatctttgt ttgattgtat tgatcaaact cttcagcaga gagtgatcga     420
    gacgtacata tctcgattat accagcctca acttgtgaag gatagtatcc agctgaaata     480
 35 tcaggattct gctgttatcg ctttatggga attcaacgaa gggcatcctg ataatagatt     540
    gggtgctatg gttatcctga agtctctaga atctgtgtca acagcaattg gagctgctct     600
    aaaggataga tcacattatg caagctctgc gggtaacaca gtgcatattg ttttgttgga     660
    tgctgatact cagacgaata caactgaaga tagtggtgat aacgaccaag ctcaagacag     720
    gatggacaaa ctctctgtaa tactgaaaca agatattgtc acggctgatc tatgtgctgc     780
 40 tggtgtcaag gttattagtt gcattgttca aagagatgga gcactcatgc ctatgcgccg     840
```

27

```
taccttcctc ttgtctgaag aaaagctttg ttatgaggaa gagccgattc ttcggcatgt      900
ggagcctcca ctttctgcac ttcttgagtt ggataaattg aaagtgaaag gatacaatga      960
gatgaagtat acaccgtcac gtgatcgtca gtggcatata tacacactta gaaatactga     1020
aaaccccaaa atgttgcaca gggtattttt ccgaactctt gtcagacagc cgagtgctgg     1080
caacaggttt atgtcaggcc atatcaatga tgttgaagta ggacatgctg aggaatctct     1140
ttcatttaca tcaagcagca tattaagatc tttgatgact gctatagaag aattggagct     1200
tcacgcgatt aggactggtc attctcatat gtacttgtgc atattgaaag agcaaaagct     1260
tcttgacctt gttcctgttt cagggaacac agttgtggat gttggccaag atgaagctac     1320
tgcgtgctct cttatgaaag aaatggctct aaagatacat gaacttgttg gtgcaagaat     1380
gcatcatctt tctgtatgcc agtgggaagt gaaacttaag ttggacagtg atggacctgc     1440
cagtggtagc tggagagttg taacaaccaa tgtcactagt cacacctgca ctgtggatat     1500
ctaccgggag gtcgaagata cagattcaca gaagctagta taccactatg ctgcatcgtc     1560
acctggtcct ttgcatggtg tcacattgag taattcatac cagcctttga gcgttattga     1620
tctaaaacga tgctctgcca ggaacaacag aactacatac tgctacgatt ttccattggc     1680
atttgaaact gcagtgcgga agtcatgggc taacgtttct agtgaaaaca accaatgtta     1740
tgttaaagca acagagctgg tgtttgctga caagaatggg tcgtggggca ctcctatagt     1800
tcctatgcag cgtgctgctg ggctcaatga catcggtatg gttgcgtgga tcttggacat     1860
gtccactcct gaatttccca gtggcagaca gattattgtt atcgcaaatg atattacttt     1920
cagagctgga tcatttggcc caagggaaga tgcatttttt gaagctgtta ccaacctggc     1980
ttgtgagaga aagcttcctc ttatctacat ggcagcaaac tctggtgctc ggattggcat     2040
tgctgatgaa gtaaaatctt gcttccgtgt tgaatgggtt gatcctgcca accctgaacg     2100
tggatttaag tacatttatc tgaatgaaga agactatggt cgtattagct cttctgttat     2160
agcgcacaag acacagctag atagtggcga ataaggtgg gttatcgatt ctgttgtggg     2220
caaggaggat ggactaggtg tggagaacat acatggaagt gctgctattg ccagtgctta     2280
ttctagggca tacgaggaga catttacact tacatttgtg agtggtcgaa cggttggaat     2340
aggagcctat cttgctcggc ttggcatacg gtgcatacag cgtattgacc agcccattat     2400
cttaactggg ttttctgccc tgaacaagct tctcgggcgg gaagtgtaca gctcccacat     2460
gcagttgggt ggtcccaaaa tcatggctac aaatggtgtt gtccatctga ctgttccaga     2520
tgaccttgaa ggtgtttcta atatattgag gtggctcagt tatgttcctg ctaacattgg     2580
tggacctctt cctattacaa aatctttaga cccaatagac agacctgttg catacatccc     2640
tgagaataca tgtgatcctc gtgcagccat cagtggcatt gatgacagcc aagggaaatg     2700
gttgggtggt atgtttgaca aagacagttt tgtggagaca tttgaaggat gggcaaagac     2760
agtagttact ggcagagcaa aacttggagg gattcctgtg ggtgttatag ctgtggagac     2820
acagaccatg atgcagctcg tccctgcaga tccaggccag cctgattccc atgagcggtc     2880
tgttcctcgt gcggggcaag tctggtttcc agattcagct accaagacag cgcacgcaat     2940
gttggacttc aaccgtgaag gattacctct gttcatcctt gctaactgga gaggcttctc     3000
tggtgggcaa agagaccttt ttgaaggaat t                                    3031
```

        <210> 24

40   <211> 1010

```
        <212> PRT
        <213> Avena sativa


        <400> 24
 5 Gln Asn Ser Lys Asp Leu Gln Lys Val Val Asp Ile Val Leu Ser His
     1               5                  10                  15
   Gln Gly Val Arg Ser Lys Thr Lys Leu Ile Leu Thr Leu Met Glu Lys
                  20                  25                  30
   Leu Val Tyr Pro Asn Pro Ala Ala Tyr Arg Asp Gln Leu Ile Arg Phe
10            35                  40                  45
   Ser Ser Leu Asn His Lys Arg Tyr Tyr Lys Leu Ala Leu Lys Ala Ser
              50                  55                  60
   Glu Leu Leu Glu Gln Thr Lys Leu Ser Glu Leu Arg Thr Ser Ile Val
   65                  70                  75                  80
15 Arg Asn Leu Ser Ala Leu Glu Met Phe Thr Glu Glu Arg Ala Gly Phe
                  85                  90                  95
   Ser Leu Gln Glu Lys Lys Leu Ala Ile Asn Glu Ser Met Gly Asp Leu
                 100                 105                 110
   Val Ser Ala Pro Leu Pro Val Glu Asp Ala Leu Ile Ser Leu Phe Asp
20           115                 120                 125
   Cys Ile Asp Gln Thr Leu Gln Gln Arg Val Ile Glu Thr Tyr Ile Ser
             130                 135                 140
   Arg Leu Tyr Gln Pro Gln Leu Val Lys Asp Ser Ile Gln Leu Lys Tyr
   145                 150                 155                 160
25 Gln Asp Ser Ala Val Ile Ala Leu Trp Glu Phe Asn Glu Gly His Pro
                 165                 170                 175
   Asp Asn Arg Leu Gly Ala Met Val Ile Leu Lys Ser Leu Glu Ser Val
                 180                 185                 190
   Ser Thr Ala Ile Gly Ala Ala Leu Lys Asp Arg Ser His Tyr Ala Ser
30           195                 200                 205
   Ser Ala Gly Asn Thr Val His Ile Val Leu Leu Asp Ala Asp Thr Gln
             210                 215                 220
   Thr Asn Thr Thr Glu Asp Ser Gly Asp Asn Asp Gln Ala Gln Asp Arg
   225                 230                 235                 240
35 Met Asp Lys Leu Ser Val Ile Leu Lys Gln Asp Ile Val Thr Ala Asp
                 245                 250                 255
   Leu Cys Ala Ala Gly Val Lys Val Ile Ser Cys Ile Val Gln Arg Asp
                 260                 265                 270
   Gly Ala Leu Met Pro Met Arg Arg Thr Phe Leu Leu Ser Glu Glu Lys
40           275                 280                 285
```

29

```
   Leu Cys Tyr Glu Glu Glu Pro Ile Leu Arg His Val Glu Pro Pro Leu
       290                 295             300
   Ser Ala Leu Leu Glu Leu Asp Lys Leu Lys Val Lys Gly Tyr Asn Glu
       305             310             315             320
 5 Met Lys Tyr Thr Pro Ser Arg Asp Arg Gln Trp His Ile Tyr Thr Leu
                   325             330             335
   Arg Asn Thr Glu Asn Pro Lys Met Leu His Arg Val Phe Phe Arg Thr
                   340             345             350
   Leu Val Arg Gln Pro Ser Ala Gly Asn Arg Phe Met Ser Gly His Ile
10         355             360             365
   Asn Asp Val Glu Val Gly His Ala Glu Glu Ser Leu Ser Phe Thr Ser
       370             375             380
   Ser Ser Ile Leu Arg Ser Leu Met Thr Ala Ile Glu Glu Leu Glu Leu
       385             390             395             400
15 His Ala Ile Arg Thr Gly His Ser His Met Tyr Leu Cys Ile Leu Lys
                   405             410             415
   Glu Gln Lys Leu Leu Asp Leu Val Pro Val Ser Gly Asn Thr Val Val
                   420             425             430
   Asp Val Gly Gln Asp Glu Ala Thr Ala Cys Ser Leu Met Lys Glu Met
20         435             440             445
   Ala Leu Lys Ile His Glu Leu Val Gly Ala Arg Met His His Leu Ser
       450             455             460
   Val Cys Gln Trp Glu Val Lys Leu Lys Leu Asp Ser Asp Gly Pro Ala
   465             470             475             480
25 Ser Gly Ser Trp Arg Val Val Thr Thr Asn Val Thr Ser His Thr Cys
                   485             490             495
   Thr Val Asp Ile Tyr Arg Glu Val Glu Asp Thr Asp Ser Gln Lys Leu
               500             505             510
   Val Tyr His Tyr Ala Ala Ser Ser Pro Gly Pro Leu His Gly Val Thr
30         515             520             525
   Leu Ser Asn Ser Tyr Gln Pro Leu Ser Val Ile Asp Leu Lys Arg Cys
       530             535             540
   Ser Ala Arg Asn Asn Arg Thr Thr Tyr Cys Tyr Asp Phe Pro Leu Ala
       545             550             555             560
35 Phe Glu Thr Ala Val Arg Lys Ser Trp Ala Asn Val Ser Ser Glu Asn
                   565             570             575
   Asn Gln Cys Tyr Val Lys Ala Thr Glu Leu Val Phe Ala Asp Lys Asn
               580             585             590
   Gly Ser Trp Gly Thr Pro Ile Val Pro Met Gln Arg Ala Ala Gly Leu
40         595             600             605
```

30

```
   Asn Asp Ile Gly Met Val Ala Trp Ile Leu Asp Met Ser Thr Pro Glu
           610             615             620
   Phe Pro Ser Gly Arg Gln Ile Ile Val Ile Ala Asn Asp Ile Thr Phe
   625             630             635             640
 5 Arg Ala Gly Ser Phe Gly Pro Arg Glu Asp Ala Phe Phe Glu Ala Val
                   645             650             655
   Thr Asn Leu Ala Cys Glu Arg Lys Leu Pro Leu Ile Tyr Met Ala Ala
               660             665             670
   Asn Ser Gly Ala Arg Ile Gly Ile Ala Asp Glu Val Lys Ser Cys Phe
10         675             680             685
   Arg Val Glu Trp Val Asp Pro Ala Asn Pro Glu Arg Gly Phe Lys Tyr
           690             695             700
   Ile Tyr Leu Asn Glu Glu Asp Tyr Gly Arg Ile Ser Ser Ser Val Ile
   705             710             715             720
15 Ala His Lys Thr Gln Leu Asp Ser Gly Glu Ile Arg Trp Val Ile Asp
               725             730             735
   Ser Val Val Gly Lys Glu Asp Gly Leu Gly Val Glu Asn Ile His Gly
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    945                 950                 955                 960
  5 Val Pro Arg Ala Gly Gln Val Trp Phe Pro Asp Ser Ala Thr Lys Thr
                        965                 970                 975
    Ala His Ala Met Leu Asp Phe Asn Arg Glu Gly Leu Pro Leu Phe Ile
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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N9/00   C12N15/82   A01H5/00   A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL EBI Accession No: 048959, 1 June 1998 (1998-06-01) GORNICKI P. ET AL.: "ACETYL-COENZYME A CARBOXYLASE; from Triticum Aestivum" XP002122229 see sequence abstract --- -/--	1-50

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 November 1999	26/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chakravarty, A

International Application No

PL./US 99/14022

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL EBI Accession No. Q08367, 1 November 1996 (1996-11-01) ASHTON A.R. ET AL.: "ACETYL-COA CARBOXYLASE (ACC); from Zea Mays" XP002122230 see sequence abstract ---	1-50
X	DATABASE EMBL EBI Accession no. AF029895, 3 February 1998 (1998-02-03) GORNICKI P. ET AL.: "Triticum aestivum acetyl-coenzyme A carboxylase (Acc-1) mRNA, nuclear gene encoding plastid protein, complete cds." XP002122231 see sequence abstract ---	1-50
X	DATABASE EMBL EBI Accession no. U58598, 31 May 1996 (1996-05-31) ASHTON A.R. ET AL.: "Zea mays acetyl CoA carboxylase mRNA, partial cds." XP002122232 see sequence abstract ---	1-50
X	WO 93 11343 A (GAHAN JOHN PETER) 10 June 1993 (1993-06-10) figure 3 ---	1-50
X	WO 96 31609 A (UNIV MINNESOTA ;GENGENBACH BURLE G (US); SOMERS DAVID A (US); WYSE) 10 October 1996 (1996-10-10) the whole document -----	1-50

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9311343	A	10-06-1993	AU	2937192 A	28-06-1993
WO 9631609	A	10-10-1996	BR	9608013 A	02-03-1999
			CA	2217367 A	10-10-1996
			EP	0820513 A	28-01-1998